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Aberrant kinase activation as a therapy target: Chronic myeloid leukemia as a model disease

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ACADEMIC DISSERTATION

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“To my family”

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ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I-IV):

I Mustjoki S, Hernesniemi S, Rauhala A, Kahkonen M, Almqvist A, Lundan T, Porkka K. A novel dasatinib-sensitive RCSD1-ABL1 fusion transcript in chemotherapy-refractory adult pre-B lymphoblastic leukemia with t(1;9)(q24;q34). *Haematologica.* 2009;94(10):1469-71

II Jalkanen S (Hernesniemi S), Vakkila J, Kreutzman A, Nieminen JK, Porkka K, Mustjoki S. Poor cytokine-induced phosphorylation in chronic myeloid leukemia patients at diagnosis is effectively reversed by tyrosine kinase inhibitor therapy. *Experimental hematology*. 2011;39(1):102-13

III Lahesmaa-Korpinen AK, Jalkanen S (Hernesniemi S), Chen P, Valo E, Nunez-Fontarnau,J, Rantanen V, Oghabian A, Vakkila J, Porkka K, Mustjoki S, Hautaniemi S. FlowAnd: Comprehensive Computational Framework for Flow Cytometry Data Analysis. *Journal of Proteomics and Bioinformatics.* 2011; 4 (11): 245-249

IV Jalkanen S (Hernesniemi S), Lahesmaa-Korpinen AM, Heckman C, Rantanen V, Porkka K, Hautaniemi S, Mustjoki S. Phosphoprotein profiling predicts response to tyrosine kinase inhibitor therapy in chronic myeloid leukemia patients. *Experimental Hematology*. 2012; 40 (9): 705-714.

ABSTRACT

Chronic myeloid leukemia (CML) is a hematologic malignancy that originates from pluripotent hematopoietic stem cells. The genetic abnormality underlying this disease, as well as a subtype of acute lymphoblastic leukemia (ALL), is a translocation between chromosomes 9 and 22, which leads to the formation of the Philadelphia (Ph) chromosome and the oncogenic BCR-ABL1 fusion protein. This oncoprotein induces cell proliferation, causes abnormal migration, and reduces apoptosis. Targeted inhibition of the BCR-ABL1 protein with imatinib and other specific tyrosine kinase inhibitors (TKIs) has revolutionized CML treatment and is currently regarded as the gold standard of targeted cancer therapy. In this doctoral thesis, different methodological approaches were used to characterize aberrant signaling activities in leukemic and normal cells with the ultimate aim of isolating novel prognostic markers for predicting TKI therapy outcome.

First, the molecular mechanism of the disease was investigated in an ALL patient with a translocation between chromosomes 1 and 9. Cytogenetic characterization showed involvement of the *ABL1* gene fused to an unknown gene. The *RCSD1* gene was selected as a candidate translocation partner and subsequent molecular dissection confirmed a *RCSD1-ABL1* fusion. This novel fusion gene predicts sensitivity to TKI therapy in ALL patients.

Second, several phosphoproteins related to immune cell function were analyzed both from the diagnostic phase CML patients and from patients under TKI therapy in order to reveal distinct signaling patterns associated with therapy responses. A single cell phosphoprotein method based on multiparameter flow cytometry was used to analyze phosphoprotein levels in different cell populations. The responsiveness of myeloid cells to *ex vivo* cytokine stimulation in diagnostic-phase patients was low, but was normalized in TKI-treated patients. In general, the blood leukocyte subsets responded normally to various cytokine stimulations, which indicated a non-immunosuppressive role for TKIs. This project also led to the development of a software–assisted analysis program, which can conduct the whole range of flow cytometry data analysis, thereby diminishing the effort needed for manual cell population gating and interpretation.

Lastly, biomarkers for therapy response were identified by analyzing aberrant signaling activities in leukemic cells collected at the time of diagnosis. The analysis was based on a phosphoproteomic array measuring phosphorylation of 46 different kinase targets. Several proteins had aberrant phosphorylation levels in patients with a poor therapy outcome – pSTAT5b being the most prominent. These biomarkers could be useful in guiding therapy selection at the time of diagnosis.

In conclusion, the findings imply that TKI therapy responses can be linked to certain biological markers, which can possibly be utilized in clinical applications in the future.

ABBREVIATIONS

A488	Alexa488
ABL1	Abelson murine leukemia viral oncogene homolog 1 gene
AML	Acute myeloid leukemia
ALL	Acute lymphoblastic leukemia
APC-H7	Allophycocyanin H7
ATP	Adenosine triphosphate
BCR	Breakpoint cluster region gene
BCR	Breakpoint cluster region
BIM	BCL2-like protein coding gene
BM	Bone marrow
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CC	Coiled coil domain
CCyR	Cytogetic response
CHR	Complete hematologic response
CML	Chronic myeloid leukemia
CMR	Complete molecular response
DDR1	Discoidin domain receptor 1
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonuclec acid
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal- regulated kinase
EUTOS	European treatment and outcome Study
FACS	Fluorescent-activated cell sorting
FBS	Fetal bovine serum
FDA	Food and Drug administration
FISH	Fluorescence *in situ* hybridisation
FSC	Forward scatter
GIST	Gastrointestinal tumor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GRB2	Growth factor receptor –bound protein 2
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
IFN-α	Interferon alpha
IFN-γ	Interferon gamma
IL	Interleukin
IS	International scale
JAK	Janus kinase
KD	Kinase domain
MAPK	Mitogen-activated protein kinase
MMR	Major molecular response
MNC	Mononuclear cell
MDR1	Multi drug resistance protein 1

MRD	Minimal residual disease
NSCLC	Non-small-cell lung cancer
PB	Peripheral blood
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
PE	Phycoerythrin
PE-Cy7	PE-Cyanine7
PerCP	Peridinin chlorophyll protein
Ph	Philadelphia chromosome
PKC	Protein kinase C
PLCγ-1	Phospholipase C γ-1
RQ-PCR	Real time quantitative polymerase chain reaction
SCNP	Single-cell network profiling
SH2	SRC Homology 2 domain
SSC	Side scatter
STAT	Signal transducer and activator of transcription
STK	Serine/Threonine kinase
TKI	Tyrosine kinase inhibitor
Treg	Regulatory T cell
VEGFR	Vascular endothelial growth factor receptor

1 INTRODUCTION

Cancer is a heterogeneous group of diseases characterized by the accumulation of genetic changes in tumor cells giving them a growth advantage over normal cells and resulting in uncontrollable cell proliferation. Leukemia is a type of cancer that affects leukocytes of the blood or bone marrow (BM). The expanding leukocytes are usually immature, based on their developmental stage. Leukemias are classified as either myeloid or lymphatic by their cell lineage, as well as acute or chronic by the nature of progression.

Protein kinases regulate cellular signaling cascades as a response to extracellular and intracellular stimuli, thus controlling cell survival, proliferation, cell growth and other cellular processes. Kinases function by transferring phosphate molecules to their substrates in a process called phosphorylation. They can be divided into cell surface receptor and intracellular non-receptor kinases as well as tyrosine and serine/threonine kinases based on substrate specificity. Deregulated kinase signaling due to various mechanisms can disrupt cell homeostasis and lead to cancer. At present, the cause of pathogenesis of many cancers is known to be aberrant, abnormally controlled kinase signaling, which has guided the development of targeted cancer therapies.

Chronic myeloid leukemia (CML) is one of the most studied types of cancer, and has for decades been regarded as a model disease in cancer research. The pathogenesis of CML and a subtype of acute lymphoblastic leukemia (ALL) is characterized by the formation of the Philadelphia (Ph) chromosome, a reciprocal translocation between chromosomes 9 and 22 t(9;22)(q34;q11) occurring in hematopoietic stem cells. The Ph chromosome contains an oncogenic *BCR-ABL1* fusion gene that encodes for a constitutively active BCR-ABL1 fusion protein that is responsible for increased proliferation, abnormal migration, and reduced apoptosis.

Imatinib (Glivec, Novartis Pharma) was the first tyrosine kinase inhibitor (TKI) developed specifically to combat the increased tyrosine activity of the BCR-ABL1, and has since been considered the model example for targeted cancer therapy. Imatinib renders the kinase domain (KD) of BCR-ABL1 dysfunctional, thereby inhibiting the oncogenic signaling that protects cancerous cells from apoptosis. Due to major improvements in the therapy responses after introduction of imatinib, CML is now regarded as a chronic, manageable disease. However, some patients develop resistance to imatinib, resulting in disease relapse. The majority of resistance can be explained by the occurrence of point mutations in the KD of BCR-ABL1, which prevents efficient binding of imatinib. To tackle this problem, a series of second and third generation TKIs have been developed that have shown promising results with many of these patients. Some therapy failures are not associated with mutations but other defined resistance mechanisms.

The presence of *BCR-ABL1* fusion gene transcript in blood or BM is the basis for measuring therapy response for Ph+ leukemias. In the TKI era, the polymerase chain reaction (PCR) has provided the most sensitive method for evaluating therapy

responses, as well as in detecting point mutations. A notable area of research deals with defining valid biomarkers for the guidance in selecting the optimal TKI therapy. The ability to find these candidate markers are based on genetic, proteomic, and immunological factors. Despite the good responses obtained with TKIs, CML is not regarded as a curable disease at this point in time. Therefore, in addition to BCR-ABL1, new therapy targets maybe required in the cure for CML patients.

This study was aimed to understand the molecular background of a distinct form of Ph-negative ALL disease, which was empirically shown to be sensitive for TKI therapy. In addition, different methodological approaches were used to characterize cellular signaling patterns in leukemic and non-leukemic cells in CML patients at diagnosis and during TKI therapy and their correlation with therapy responses was evaluated. Finally, an automated flow cytometry data analysis program was developed to assist time-consuming and laborious flow cytometry data analysis.

2 REVIEW OF THE LITERATURE

2.1 Kinase signaling as a therapy target

2.1.1 Kinase signaling at the molecular level

Protein kinases are a large family of enzymes that are essential for mediating external stimuli inside cells. They are divided into serine/threonine kinases (STK) and tyrosine kinases (TK) according to their substrate specificity. [1] TKs activate numerous signaling pathways that are responsible for cell proliferation, differentiation, migration, and metabolic changes. Due to these functions TKs have well-established role in many cancers and other proliferative diseases. [2] STKs phosphorylate transcription factors, cell cycle regulators cytoplasmic and nuclear effectors, and are linked to cancer as well.[3]

Kinases also fall into two categories according to their location and function inside the cell. Receptor kinases are transmembrane proteins with an extracellular ligand-binding domain. The intracellular domain is responsible for catalytic activity. Nonreceptor kinases reside in the cytosol, nucleus, and inner surface of the plasma membrane and therefore lack a transmembrane domain. Activity of both types of kinases is regulated tightly, so that normal cells in a resting state have low levels of phosphorylated residues. [4]

The core kinase structure responsible for adenosine triphosphate (ATP) binding is highly conserved in eukaryotic kinases [5]. This core structure is built from the N-terminal lobe and a larger C-terminal lobe connected with a hinge region. ATP binds to a cleft between the two lobes, while substrate binds to the C-terminal lobe. The adenine ring of ATP forms hydrogen bonds with the hinge region and the glycine loop in the N-terminal lobe binds to the triphosphate. An activation loop in the C-terminal lobe includes conserved DFG and APE sequences. In the active conformation, the activation loop is usually phosphorylated and in the catalytically active state. The inactive state conformation of the activation loop has a higher variation between kinases and blocks the substrate-binding site. [6]

In their inactive form, receptor TKs are monomeric or dimeric and unphosphorylated. Binding of a specific ligand to the extracellular domain triggers receptor dimerization, stabilization of the dimer, and transphosphorylation of tyrosine residues in the activation loop. This generates recruitment sites for downstream signaling molecules that have phosphotyrosine recognitions domains. [7] These include either the SRC homology-2 (SH2) domain or the phosphotyrosine binding (PTB) domain. [8] The tyrosine phosphorylation of the receptor kinases also activates the enzymatic TK activity. [7] In addition to the activation loop, a subset of receptor TKs have a juxtamembrane region in the C-terminal tail. This region has autoinhibitory properties, which prevent the TK function by interacting with the KD in the inactive state. [9,10]

Intracellular kinases are regulated and maintained inactive trough interactions with inhibitory proteins, lipids, and autoinhibitory mechanisms. Activation is mediated by intracellular signals such as dissociation of inhibitors, by interaction with transmembrane receptors, and by phosphorylation by other kinases.[4]

2.1.2 Kinase inhibition in cancer therapy

Since the discovery of the first oncogenic kinase, v-SRC in 1970, [11] deregulated kinase signaling has been linked to pathogenesis of cancer. In addition to cancers, deregulated kinases are associated with various immunological, metabolic, neurological, and infectious diseases. Despite the characterization of this clear connection between molecular level defects and disease, not much effort was initially made to target kinases. [12] Eventually, the discovery of staurosporine, a protein kinase C (PKC) inhibitor naturally produced by *Streptomyces,* [13] led to activation of research in the field of kinase inhibitors.

Small molecule kinase inhibitors are today the most popular type of cancer drug under development in the pharmaceutical industry. The discovery of new kinase inhibitors has benefited enormously from rational drug design through crystallographic techniques. The assessment of kinase inhibitor selectivity has also improved with modern proteomic approaches. This kind of progress made in the technologies of inhibitor development has ensured fast discovery of selective small molecule inhibitors with optimal pharmacological properties.

In 2010, 149 kinase inhibitors were being tested in clinical trials (Fig. 1), [14] and by 2012, 13 small-molecule kinase inhibitors were approved for treatment of cancer by the U.S. Food and Drug Administration (FDA) (Table 1). [15] The targets of these inhibitors included ABL1 (BCR-ABL1), platelet-derived growth factor receptor (PDGFR), KIT kinases, as well as the endothelial growth factor receptor (EGFR) and vascular endothelial growth factor receptor (VEGFR) families. [16]

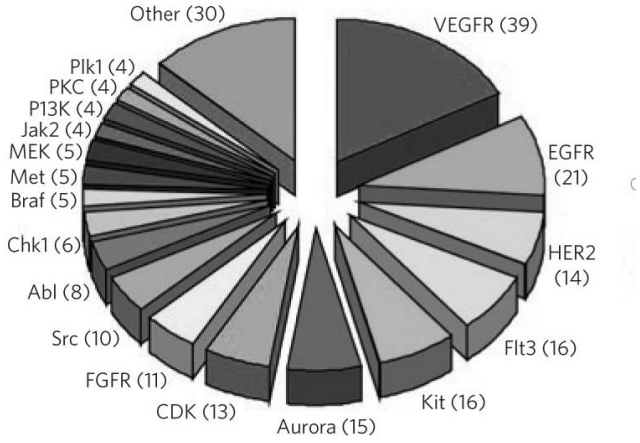

Figure 1. *Kinase targets in clinical trials in 2010. Reprinted with permission. © 2010 Nature Publishing Group.*[14]

Table 1. *FDA-approved small molecule kinase inhibitors for clinical use. Adjusted from Dar AC & Shokat KM 2011[15] and Jänne PA et al. 2009 [16]*

Inhibitor	Main kinase targets	Indication	FDA approval year
Imatinib	BCR-ABL1, C-KIT, PDGFR	CML, Ph+ALL, GIST, HES*	2001
Gefitinib	EGFR, ERBB2, HER4	NSCLC	2003
Sorafenib	C-KIT, PDGFR, VEGFR	Renal cancer, hepatocellular carcinoma	2005
Erlotinib	EGFR, ERBB2	NSCLC, pancreas cancer	2005
Sunitinib	C-KIT, PDGFR, VEGFR	Renal cancer, imatinib-refractory GIST, pancreatic neuroendocrine tumor	2006
Dasatinib	BCR-ABL1, SRC family	CML and Ph+ ALL	2006
Nilotinib	BCR-ABL, PDGFR, C-KIT	CML	2007
Lapatinib	ERBB2, EGFR	HER2+ breast cancer	2007
Pazopanib	VEGFR, PDGFR, C-KIT	Renal cell carcinoma, soft tissue carcinoma	2009
Vemurafenib	B-RAF	Melanoma	2011
Ruxolitinib	JAK	Myelofibrosis	2011
Axitinib	VEGFR, PDGFR, C-KIT	Renal cell carcinoma	2012
Bosutinib	BCR-ABL1, SRC	CML resistant to other therapies	2012

*CML, chronic myeloid leukemia; GIST, gastrointestinal tumor; HES, hypereosinophilic syndrome; ALL, acute lymphoblastic leukemia; NSCLC, non-small-cell lung cancer; PDGFR, platelet-derived growth factor receptor; EGFR, epidermal growth factor receptor; C-KIT, CD117, mast/stem cell growth factor receptor; ERBB2, human epidermal growth factor receptor 2 (HER2); JAK, janus kinase; BRAF, serine/threonine kinase encoded by v-RAF murine sarcoma viral oncogene homolog B, *also for dermatofibrosarcoma protuberans, systemic mastocytosis.*

2.1.2.1 Types of kinase targets

The efficacy of kinase inhibition is most prominent when cancer is driven by a single alteration in a kinase essential for cancer cell proliferation and survival. This is termed 'oncogene addiction' and renders cancer cells susceptible to targeted inhibition of the oncogenic kinase. [17,18] Another type of kinase inhibition targets kinases that are encoded by a pair of synthetic lethal genes, which are lethal when both genes are mutated. [19] When only one gene of the pair is mutated, targeting of the nonmutated kinase would result in a synthetic lethal phenotype. [20] These kinases are usually situated downstream of key elements in the oncogenic signaling pathways and are important for cancer cell survival and proliferation. A third class of kinase targets is essential for maintaining the tumor microenvironment or its kinases are expressed within the tumor itself and are required for formation and maintenance of the tumor. At present, the first type of kinases – those based on kinase addiction – are the most potent targets for kinase inhibition. These kinases are usually highly transforming, easily identifiable by DNA sequencing, and the inhibitor screening is somewhat straightforward during optimization. [12]

2.1.3 Kinase inhibitors

The strategy underlying the use of kinase inhibitors is to prevent transfer of the terminal phosphate of ATP to tyrosine, serine, or threonine residues of the substrate molecule. The majority of kinase inhibitors are ATP competitive and small-weight molecules. [12] Type I inhibitors preferentially bind to the active conformation of the kinase and are the most common type of ATP competitive inhibitors. They mimic the hydrogen bonds normally formed between the adenine ring and the hinge region in the core structure. [21,22] Type II inhibitors are characterized by their binding to the inactive conformation of the kinase. In the inactive conformation, the activation loop DFG sequence points outward, exposing the hydrophobic site adjacent to the ATP binding site. Examples of these types of inhibitors are imatinib and nilotinib, which inhibit BCR-ABL1 but also several other kinases such as C-KIT. [23] The crystal structure of the complex formed by this type inhibitor and the kinase was first revealed with imatinib bound to ABL1. [24] The type II kinase inhibitor binding site is less conserved and therefore has a higher potential for specific inhibition than do the type I inhibitors. [21,25]

A completely different mechanism for targeting kinases is taken by allosteric inhibitors, which are uncompetitive with ATP binding. Non-ATP competitive inhibitors are also called type III inhibitors. These bind to an allosteric site, which can bind regulators that affect enzyme activity. The binding sites show high variation between different kinases, resulting in the observation of high selectivity with allosteric inhibitors. [12,26] Covalent inhibitors are the fourth type of kinase inhibitors; these form irreversible covalent bonds with the active site. The covalent bond is usually formed between the inhibitor and a cysteine residue, resulting in blockage of the ATP binding site. [27] The advantage of these inhibitors is the possibility of having a short half-life, combined with reduced dosing. However, concerns have been raised regarding the adverse effects caused by off-target irreversible complexes formed by covalent inhibitors. [28]

2.1.4 Selectivity of kinase inhibitors

The goal of developing kinase inhibitors is to achieve the highest possible selectivity against a specific kinase. However, many clinically approved inhibitors inhibit a number of other kinases. [29] The challenge for the development of specificity comes not only from the 518 kinases of the human kinome, [30] but also from large number of other purine-binding proteins coded by the human genome. [31] The advantage of selective kinase inhibitors is their smaller likelihood of toxicity, whereas inhibitors with a broader target spectrum can be utilized with multiple indications and can simultaneously affect many cellular processes associated with the tumor. [16]

Several methods are available for assessment of the *in vitro* selectivity of inhibitors. These assays are based on measurement of the phosphorylation, dissociation constant, and melting temperature of the inhibited kinase. [32] At the moment, selectivity data are accessible for 178 commercially available kinase inhibitors that function against 300 kinases. [33] The selectivity of kinase inhibitors should also be evaluated at the cellular level, because *in vitro* results do not always apply *in vivo*. A popular system for studying kinase inhibitor selectivity at the cellular level is the murine interleukin-3 (IL-3) dependent pro-B cell line (BA/F3), which can be transformed with oncogenes and in which the ability of kinase inhibitor to induce specific cell death can be discriminated from non-specific cell death by addition of IL-3. [34] A chemical genomics method utilizes yeast cells expressing engineered kinases, in which specific kinase inhibition is observed as a transcriptional change.[35] A high throughput assay uses HEK-293 cells and fluorescence resonance energy transfer (FRET), where kinase inhibitor binding causes a conformational change in the kinase and elicits a detectable signal. [36] The transition from the cell level to the organismal level might also affect the efficacy of a kinase inhibitor due to various factors such as differential kinase expression and drug distribution in different tissues. [32]

2.1.5 Factors behind the sensitivity of kinase inhibition

An increasing challenge in the clinic is to overcome resistance to kinase inhibition therapy. *De novo* resistance describes a situation whereby the response to an inhibitor is not achieved in the initial treatment. The reasons behind this can be lack of addiction to the kinase inhibited and altered drug metabolism, which are regulated by germ line polymorphisms. Acquired resistance occurs when the response to a particular kinase inhibitor is lost during the course of the treatment. [16] The mechanism of acquired resistance can be assessed with genomic analysis of samples from kinase inhibitor resistant patients. Long-term cancer cell culture with a kinase inhibitor can result in the development of resistant clones, which can then be used to reveal the mechanism underlying resistance. [37]

The major cause for acquired resistance is point mutations in the KD area. A common point mutation results in replacement of the gatekeeper residue threonine into a larger hydrophobic residue. This gatekeeper residue controls the access of kinase inhibitors to the hydrophobic pocket deep within the active site. Point mutations that result in amino acid residues with bulky side chains at this particular site prevent the binding of kinase inhibitors, while catalytic activity remains or is even stimulated. [38] Another example of inhibitor resistance mechanism involves amplification of the *MET* gene in

EGFR-activated cancers. MET signaling activates survival signaling in a fashion similar to that seen with EGFR signaling, and renders EGFR inhibition ineffective. This is regarded as a compensatory circuit pathway. [39] Elevated levels of the MET ligand hepatocyte growth factor (HGF) have a similar effect to that seen with amplification of the *MET* gene. [40]

Acquired resistance involving mutations in the KD has been overcome by rational design of second-generation inhibitors that are able to inhibit the mutated kinase. Combination therapies, instead of monotherapies, are also an ideal option for treatment of cancers, in which compensatory signaling pathways have developed as a survival mechanism. Genetic screening at the time of diagnosis for polymorphisms known to affect kinase inhibitor efficacy can possibly be used in the future to guide therapy selection and dosage. [16]

2.2 Chronic myeloid leukemia as a model disease of kinase inhibition therapy

2.2.1 The Ph chromosome behind the pathogenesis of CML

Chronic myeloid leukemia is one of the most extensively studied human malignancies. The cause for the disease was found in 1960, [41] when the Ph chromosome was discovered, and reciprocal translocation between chromosomes 9 and 22 was revealed in 1973. [42] In the 1980s, the molecular mechanism of the disease was identified when the t(9:22) translocation was shown to involve the Abelson murine leukemia viral oncogene homolog 1 gene (*ABL1*) from chromosome 9 [43] and a breakpoint cluster region (*BCR*) from chromosome 22 [44] (Fig. 2). Subsequently, *BCR-ABL1* fusion transcripts were discovered in patients with CML [45,46], which led to identification of the 210 kD-sized BCR-ABL1 fusion protein [47] that showed an enhanced tyrosine kinase activity compared to the normal ABL1 protein. [48] The transforming potential of BCR-ABL1 was first shown in cell lines and mouse BM cells. [49,50] The oncogenic transformation capability of the BCR-ABL1 fusion protein was later confirmed when retrovirally-infected hematopoietic stem cells expressing BCR-ABL1 were able to induce a CML-like disease in mice. [51-53]

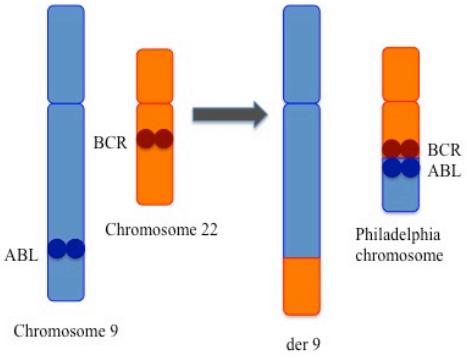

***Figure 2**. Formation of the Philadelphia chromosome in a reciprocal translocation between chromosomes 9 and 22, resulting in the BCR-ABL1 fusion gene. Modified from Hazlehurst et al. 2009.* [54]

2.2.2 The *BCR-ABL1* fusion gene

The BCR-ABL1 protein exists in a few different-sized isoforms, depending on the site of the breaking point in the *BCR* gene, and these isoforms are associated with specific types of leukemia (Fig. 3). In the majority of cases of CML, and in one third of Ph+ ALL, the BCR breaking point is situated between exons 12 to 16 (formerly b1-b5) and therefore this is referred to as the major breaking point area (M-BCR). The resulting b2a2 and b3a2 mRNAs are translated into p210 protein. The rest of the Ph+ ALL cases are comprised of a BCR-ABL1 sized p190 and encode an e1a2 mRNA resulting from the minor BCR (m-BCR) area. This type of isoform is rarely associated with CML. [55] The breaking point in the exon 19 micro-BCR (μ-BCR) results in a p230 isoform, which is associated with a rare neutrophilic CML. [56]

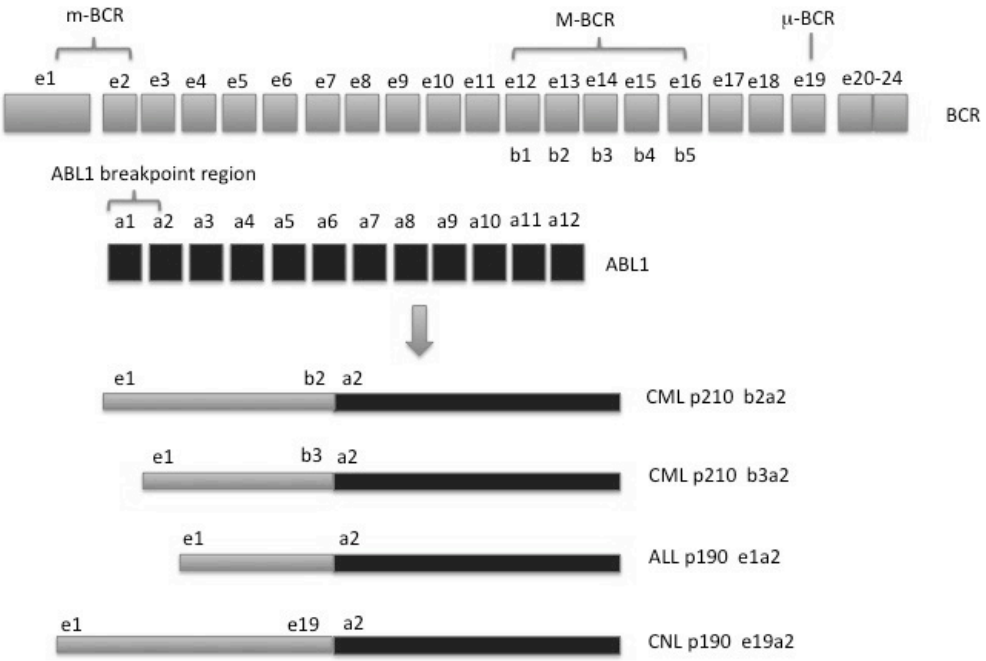

Figure 3. *Genomic structure of the BCR gene, showing three different breaking point sites: m-BCR, M-BCR and μ-BCR and ABL1 gene with a breaking point at a constant site in the upper level of the figure. The lower level of the figure represents different BCR-ABL1 mRNAs with corresponding protein sizes. Modified from Hazlehurst et al. 2009.* [54]

2.2.3 BCR-ABL1 signaling in CML

The normal counterpart of the oncoprotein is ABL1 (also known as c-ABL,), which as a TK takes part in multiple signaling pathways that mediate responses to growth factors, ionizing radiation, oxidative stress, and integrin stimulation; it is also associated with signaling during neuronal development. [57] The subcellular localization of ABL1 depends on the cell type; for example, the localization in primary hematopoietic cells is mostly cytoplasmic. [58] The localization of ABL1 in the nucleus or the cytoplasm is determined by specific signal sequences. [57] In the cytoplasm, ABL1 is mostly bound to cytoskeletal F-actin, [59] whereas the myristoyl-modified protein is associated with the inner surface of the plasma membrane. [60] Multiple different options exist for ABL1 to localize inside the cell and these reflect the variety of its roles inside the cell. These tasks include cell adhesion and motility, [61] DNA damage responses, [62] responses to microbial infection, [63] and actin remodeling. [64] ABL1 contains a special domain structure (SRC homology 3–SRC homology 2–tyrosine kinase, SH3-SH2-TK), which gives the protein a high potential for signaling capabilities.[65]

BCR is also a signaling protein, but its function is not well known. It is a 160 kD protein with STK activity and has several distinct domains. [66] BCR has GTPase activating protein (GAP) activity towards $p21^{rac}$, [67] which is abundantly expressed in human neutrophils and where it plays a role in cytoskeletal organization and superoxide production. [68] BCR also negatively regulates WNT signaling pathway [69] and participates in cellular growth factor receptor trafficking. [70] BCR-deficient mice have neutrophils that show abnormal respiratory burst activity in the form of increased reactive oxygen metabolite production, which suggests a link between BCR function and the cell type affected in CML. [71]

The BCR-ABL1 fusion protein has been studied extensively. In contrast to the ABL1, the oncogenic BCR-ABL1 is expressed solely in the cytoplasm. [58] Its mechanism of action is associated with altered cellular adhesion, activation of mitogenic pathways, inhibition of apoptosis, and degradation of physiologically important proteins. [72] The signaling potency of the BCR-ABL1 fusion protein is based on its increased diversity of protein-binding domains compared with its normal counterpart ABL1. The N-terminal side of BCR-ABL1 has a coiled-coil (CC) domain (Fig. 4), which is important for the oncogenicity of BCR-ABL1; it activates the KD in ABL1. The CC domain is also responsible for oligomerization of the fusion protein and it binds to actin fibers. Oligomerization of BCR-ABL1 is also suggested as an essential part of ABL kinase activation. [73] The second important site in the BCR region is the growth factor receptor-bound protein 2 (GRB2)-binding site, which is responsible for formation of a protein complex consisting of GRB2, son-of-sevenless (SOS), and GRB2-associated binding protein 2 (GAB2). Formation of this complex is dependent on autophosphorylation of BCR-ABL1 at Y177, which activates the oncogenic signaling cascade associated with activation of RAS and recruitment of SHP2 and phosphatidylinositol 3-kinase (PI3K). [74-76] RAS activates the mitogen-activated protein kinase (MAPK), which enhances proliferation. The phosphorylation site Y1294, located in the ABL KD, along with the SH2 domain, also contributes to activation of the RAS pathway, although the mechanism is unclear. [77] PI3K activates the AKT pathway, which promotes survival by suppressing activity of forkhead O (FOXO) transcription factors, [78] enhances proliferation through SKP2-regulated

degradation of p27[79], and activates mTOR, leading to enhanced protein translation and proliferation. [80,81] Phosphorylation of STAT5 directly by BCR-ABL1, or by mediation of JAK2 or HCK, mediates proliferation and survival. [82,83] In CML, the primitive progenitor cells have diminished adhesion capability to stromal cells and the extracellular matrix of the BM, thus losing their normal regulatory signals provided by the environment. [84] This is assumed to be mediated by a low affinity of integrin β1 to adhesive ligands. [85,86] At a signaling level, this interaction is mediated by an interaction between BCR-ABL1 and CBL, CRKL and paxillin. [87,88] BCR-ABL1 also induces the expression of growth factors IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF), [89,90] which can have further impacts on the pathogenesis of CML.

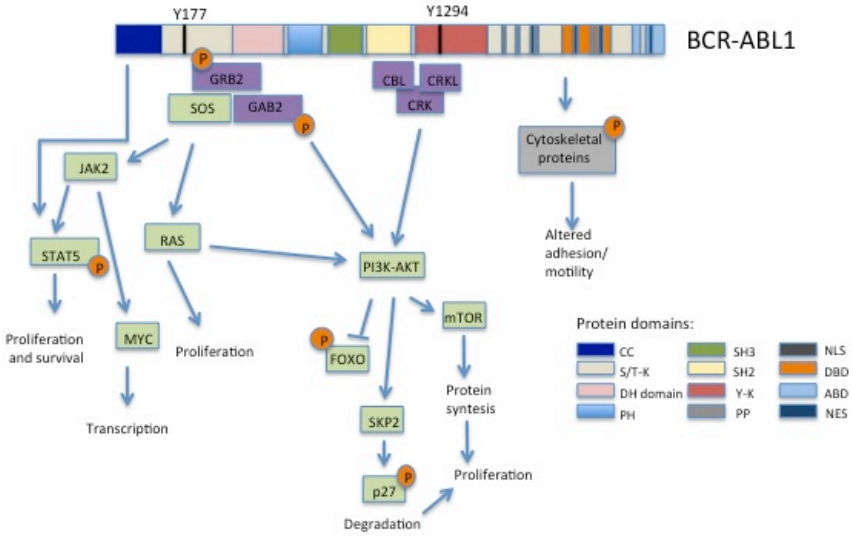

Figure 4. *Signaling pathways induced by the p210 BCR-ABL1 fusion protein. The part originated from the BCR protein includes a coiled-coil (CC) domain, serine/threonine kinase (STK), Dbl/CDC24 guanine-nucleotide exchange factor homology (DH) domain, and pleckstrin homology (PH) domain. Downstream of the breaking point are ABL1-related domains: SRC homology 3 (SH3), SH2, tyrosine kinase (TK), proline-rich SH3 binding sites (PP), nuclear localization signals (NLS), DNA-binding domain (DBD), actin-binding domain (ABD) and nuclear exporting signal (NES). Phosphorylation of tyrosine residues generates docking sites for adaptor proteins (purple), which modify activation of multiple signaling pathways affecting the cell survival, proliferation, adhesion, and motility. Modified from Ren 2005 and O´Hare et al. 2011.* [91,92]

2.2.4 Clinical characteristics and epidemiology of CML

CML is a hematologic malignancy that originates from a transformed pluripotent hematopoietic stem cell and affects the myeloid, monocytic and erythroid cells as well as platelets and B-cells, [93,94] although the clinical characteristics of CML is expansion of granulocytic-series cells in the peripheral blood (PB) (Fig. 5). Increased numbers of eosinophils, basophils, and platelets are common, as is anemia. [95] The diagnosis of CML is confirmed by the presence of the Ph chromosome and *BCR-ABL1* transcripts in the blood or BM. [96]

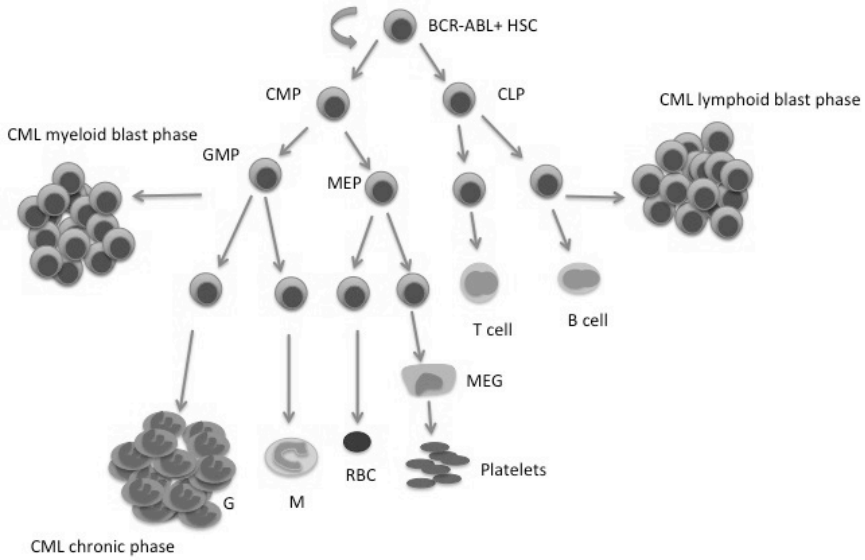

Figure 5. *Development of CML from BCR-ABL1 positive hematopoietic stem cells (HSCs). Self-renewing HSCs differentiate into myeloid cells, such as macrophages (Ms), granulocytes (Gs), red blood cells (RBCs) and platelet-producing megakaryocytes (MEGs) through common myeloid progenitors (CMPs), common granulocyte/macrophage progenitors (GMPs), and megakaryocyte/erythrocyte progenitors (MEPs). The lymphoid lineage into T and B cells occurs via a common lymphoid progenitor (CLP). Chronic myeloid leukemia (CML) is characterized by increased granulopoiesis in the chronic phase. As the disease progresses into the blast phase, more primitive cells start to accumulate. Modified from Ren 2005.*[91]

CML is usually diagnosed in the chronic phase, when patients usually lack symptoms but might experience fatigue, weight loss, and night sweats. In addition to abnormal blood counts, an enlarged spleen is a common finding of CML. CML eventually progresses to the accelerated phase, which is characterized by additional chromosomal changes, increases in total numbers of leukocytes, basophilia, thrombocytopenia, increased spleen size, and the appearance of blasts in the blood or BM. The last stage of CML is a blast crisis, which resembles acute leukemia with its

large numbers of blasts with either lymphoid or myeloid phenotype. [97] If untreated, CML will progress from the chronic phase to death within three to five years. [95]

The incidence of CML is 1 to 2 cases per 100 000; [96] however, due to progress made in the management of CML in the past decade, the number of patients with chronic phase CML is increasing. The common age at diagnosis is around 65 years; [96] it is rarely diagnosed in children [98] and the prevalence is slightly higher in men. [99]

2.2.5 Ph positive ALL and other ABL1-driven leukemias

The Ph chromosome is the most common cytogenetic abnormality seen in adult ALL patients (approximately 20%) and it contributes to 2 % of all pediatric ALL cases.[100] [101] Prognosis of Ph+ ALL was very poor prior to the introduction of TKIs as a therapy mode. [102] A *BCR-ABL1*-like gene expression profile has been recently identified among Ph negative pediatric ALL patients with B-cell precursor phenotype. This *BCR-ABL1*-like phenotype occurs in 10 % of pediatric ALL patients and is associated with a high risk of relapse. This ALL subtype is hypothesized to benefit from TKI therapy[103,104].

Other types of fusion genes involving *ABL1* have also been characterized (Fig. 6). *NUP214-ABL1* (*TEL-ABL1)* is mostly seen in T-cell ALL patients [105]. *SFPQ-ABL1* [106] and *ZMIZ1-ABL1* [107] fusions been reported in two separate B-cell ALL patients, and *EML1-ABL1* once in a T-cell ALL patient. [108] *ETV6-ABL1* is more common than previously mentioned and is seen in a heterogeneous group of hematologic malignancies. [109]

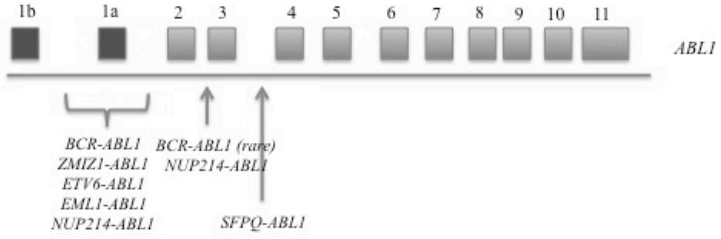

Figure 6. *ABL1 gene exons and fusion gene breaking points. Exon 1 is alternatively spliced. Modified from De Braekeleer et al. 2011.* [109]

2.2.6 Tyrosine kinase inhibitors (TKIs) in the treatment of CML

2.2.6.1 Treatment of CML before the TKI era

The history of CML reveals many attempts to treat the disease with highly toxic and unselective agents. The earliest attempts were conducted with arsenic at the end of 19th century; this treatment resulted in shrinkage of the spleen and improvement in the leukocyte count and anemia, but the response lasted only few months. [110] Radiotherapy, mainly given to the spleen, was introduced at the beginning of 20th century. This therapy mode induced a rapid decrease in spleen size and leukocyte count, and the response lasted from weeks to months and occasionally years. [111] The third major therapy mode was busulfan, which came into the clinic in the 1950s and was the treatment of choice for the next 35 years. Busulfan was found to be more effective and convenient than radiotherapy, and increased the survival of CML patients. [112,113]

Busulfan was replaced by hydroxyurea and interferon-alpha (IFN-α), which manifested less toxic effects. Hydroxyurea was more effective than busulfan in terms of prolonging survival, [114] and turned out to be an excellent debulking agent by its effective normalizing of blood counts. It is commonly used as an adjuvant prior to more specific therapy modes. Due to a lack of cytogenetic responses, hydroxyurea never gained position as a first line therapy. [115] Usage of IFN-α began in the early 1980s; it increased the survival of CML patients when compared to busulfan and hydoxyurea, and was most effective as a combination therapy with low-dose cytarabine. [116]

Allogeneic hematopoietic stem transplantation (HSCT) was tested experimentally as a treatment mode as early as 1979 [117] and provided a cure for the disease. In the 1980s, it became more popular as the HLA-matching techniques developed. [118] In a follow-up study conducted in 2005, the 15-year (patients collected 1982-92) overall survival rate was 53 % with allogeneic HSCT patients. [119] Allogeneic HSCT rates have declined since the introduction of TKI therapy for treatment of CML, but it is still an option for those patients in advanced phases and following treatment failure with TKIs. [120]

2.2.6.2 Imatinib

The specific BCR-ABL1 inhibitor, imatinib mesylate, was initially known by the names GCP 57148 or STI571 [121,122]. The lead compound, a phenylaminopyrimidine derivative, was identified in an inhibitor screening study against PKC. Chemical modifications increased the cellular activity, TK specificity, solubility, and oral bioavaibility, while decreasing unwanted PKC activity. [122] Imatinib was an efficient inhibitor of ABL1 tyrosine kinases (BCR-ABL1 and the normal counterpart) [123] as well as other kinases, such as discoidin domain receptor 1 (DDR1), PDGFR-α/β, C-KIT, ARG, and NGO2. [124]

The first indication for imatinib as a possible treatment for *BCR-ABL1* positive leukemias was indicated in preclinical studies with p210 BCR-ABL1 expressing cell lines, whose ability to proliferate were inhibited with imatinib. Imatinib also induced

a 92-98% decrease in *BCR-ABL1* positive colonies in colony-forming assays performed with primary blood and BM samples, whereas normal colony formation was unaffected. [123] An effective reduction of BCR-ABL1 phosphorylation and induction of apoptosis was visible in both BCR-ABL1 expressing cell lines and primary patient samples. [125] Imatinib also showed inhibitory activity with primary ALL samples, [125] ALL cell lines, [121,126] and TEL-ABL1 expressing cell lines. [121] The efficacy of imatinib was further confirmed in *in vivo* mice experiments in which dose-dependent inhibition of tumor growth [123], blockage of BCR-ABL1 phosphorylation, and increased tumor-free survival were observed with imatinib therapy. [127] Imatinib also corrected splenomegaly and PB leukocyte counts in mice with CML-like disease induced by retroviral transduction of transplanted BM. Leukemic cells originated from these mice also had decreased levels of phosphorylation in the BCR-ABL1 downstream molecules STAT5, CRKL, and SHC, following imatinib treatment. [128]

2.2.6.3 Mechanism of tyrosine kinase inhibition with imatinib

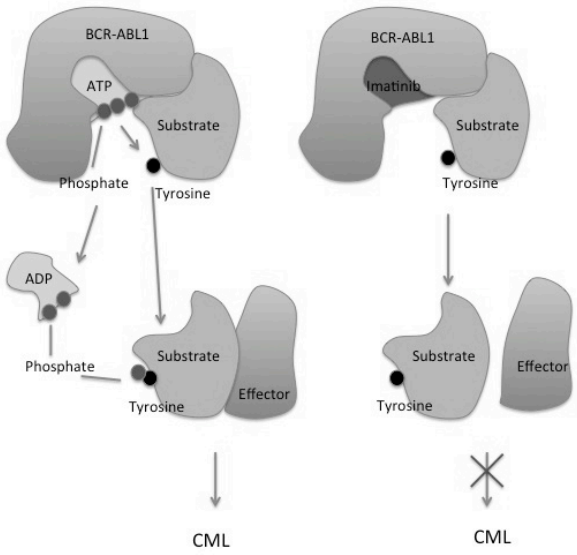

Figure 7. *Mechanism of inhibition of BCR-ABL1 function with imatinib. On the left, adenosine triphosphate (ATP) is bound to the active site of BCR-ABL1. BCR-ABL1 catalyses the phosphorylation of a tyrosine residue on the substrate molecule, which leads to activation of the signaling pathways required for generation of CML. On the right, imatinib occupies the active site of BCR-ABL1, thereby preventing phosphorylation of tyrosine residues on the substrate. Modified from Savage et al. 2002.* [129]

The mechanism of binding of imatinib to BCR-ABL1 was revealed with computational docking studies and X-ray crystallography. [122,130] Imatinib binds to the catalytically inactive form of the ABL KD and is therefore a type II inhibitor. In this inactive conformation, the activation loop is unphosphorylated and in a closed conformation and the conserved DFG motif in the N-terminal part of the loop is pointed outward. The active conformation of the activation loop would instead be open, enabling substrate binding. The inactive conformation provides specificity for imatinib, since this conformation differs from that of other kinases. The nitrogen of the pyrinidyl ring in imatinib accepts a hydrogen bond from the amide of M318 of BCR-ABL1, a bond that is normally formed with N1 nitrogen in ATP. The side chain of T315 also forms a hydrogen bond with the secondary amino group in imatinib. T315 is replaced with methionine in many other kinases, rendering the possibility to form a hydrogen bond with imatinib. This so-called gatekeeper residue is therefore essential for the specificity of imatinib to BCR-ABL1. In total, imatinib forms six different hydrogen bonds and the majority of the binding is mediated by van der Waals interactions. [24,130] Binding of imatinib prevents autophosphorylation of BCR-ABL1, thereby preventing phosphorylation of downstream signaling molecules essential for leukemogenesis, which eventually leads to inhibition of proliferation and apoptosis (Fig. 7). [123,125]

2.2.6.4 Clinical efficacy of imatinib therapy

Despite the existence of other potential kinase targets, CML was chosen as an indication for the first clinical phase I studies conducted with imatinib. The reason for this was its well-characterized pathogenesis and the oncogene addiction caused by BCR-ABL1. Monitoring of clinical responses with leukocyte counts was also highly feasible compared to solid tumors. [122] Imatinib was rapidly shown to be superior to other therapy modes, and was chosen as a first line therapy mode for CML in 2001. A phase II clinical trial was conducted with 532 IFN-α -failure CML patients; imatinib was judged as a safe and efficient therapy mode for this type of patient group after a 6-year follow-up. [131] The next phase 3 trial, the International Randomized Study of Interferon and ST1571 (IRIS) study, enrolled 1106 newly-diagnosed CML patients, who received either imatinib or IFN-α combined with cytarabine. Imatinib was shown to be superior to the IFN-α/cytarabine arm in terms of better tolerability, quality of life, and therapy response. Differences in therapy response were remarkable: the cytogenetic response rates at 18 months were 84% in the imatinib arm and 35% in the IFN-α/cytarabine arm. For this reason, the majority of patients in the IFN-α/cytarabine were switched to the imatinib arm. [132-134] Long-term follow-up of these patients at 6 years has confirmed the efficacy of imatinib therapy, since the event-free survival was 83%, freedom from progression to advanced disease was 93% and overall survival was 88%. [135]

A proportion of patients fail to respond to imatinib, which can be either due to primary resistance characterized by the absence of an initial response, or to acquired resistance, where the patient loses the response. Resistance can be further divided into hematologic, cytogenetic, and molecular resistance based on clinical and laboratory criteria. [136] Initial response rates are lower in patients with advanced-stage disease and responses tend be short-lived in this patient group compared to the responses seen in chronic phase patients. [137,138] The main reason for imatinib resistance is the existence

of various point mutations; to date, 90 different point mutations have been documented. [136,139] These mutations are more frequently associated with acquired resistance compared to primary resistance and the likelihood for detection is increased in advanced stages of the disease. [140]

Imatinib-therapy shows also efficacy among Ph+ ALL patients, [141,142] but these patients tend to progress rapidly and develop resistance [142]. Combining imatinib with chemotherapy has shown more promising results with Ph+ ALL patients preceding allogeneic HSCT. [143,144] Imatinib can be used in treatment of patients with gastrointestinal stromal tumors (GIST) who carry a mutated C-KIT, [145] hypereosinophilic syndrome or chronic eosinophilic leukemia with FIP1L1-PDGFRα fusion kinase, [146] C-KIT –driven systemic mastocytosis, [147] and chronic myeloproliferative diseases with PDGFβ rearrangements. [148] The latest indication for imatinib therapy is dermatofibrosarcoma protuberans, a rare skin cancer with deregulated expression of PDGFβ. [149]

2.2.6.5 Second generation inhibitors nilotinib and dasatinib

Dasatinib (formerly BMS-354825) has a broad kinase inhibition profile compared to imatinib, with main targets consisting of BCR-ABL1, ABL, C-KIT, PDGFR, and SRC family kinases (SRC, FGR, FYN, HCK, LCK, LYN, and YES) (Fig. 8). In total, dasatinib inhibits more than 30 different kinases. [124] The potency of dasatinib for unmutated BCR-ABL1 is over 300 times higher than that of imatinib and it was able to inhibit many mutated BCR-ABL1 isoforms that cause imatinib resistance. [150] However, some point mutations are inaccessible by dasatinib: e.g., T315I/A, F317 L/V/C, and V299L. [139,151]

The main indications for dasatinib therapy have been imatinib resistant CML and Ph+ ALL [152,153], but dasatinib has been approved also for the treatment of newly diagnosed CML patients. The SRC/ABL Tyrosine Kinase Inhibition Activity Research Trial C (START-C) evaluated the efficacy of dasatinib on CML patients who were intolerant of or resistant to imatinib. The response rates at 15 months were: complete hematologic response (CHR), 91%; major cytogenetic response (MCyR), 59%; and complete cytogenetic response (CCyR), 49%; with 96% overall survival. Therefore, dasatinib provided an efficient treatment mode for patients who were unresponsive to imatinib. [152] A phase 3 DASISION study compared dasatinib versus imatinib in newly diagnosed chronic-phase CML patients. [154] Recent results from a 2-year follow-up study showed faster and deeper responses in favor of dasatinib. [155]

Clinical trials have also shown efficacy of dasatinib in imatinib resistant and intolerant Ph+ ALL patients [156] and in newly diagnosed Ph+ ALL patients when steroids are combined as an induction therapy. [157] Assessment of dasatinib in the treatment of solid tumors such as breast cancer, prostate cancer, and melanoma is also ongoing in several clinical trials, as reviewed by Montero. [158]

Nilotinib (formerly AMN107) inhibits BCR-ABL1 with 20 times higher potency than imatinib and has the ability to inhibit imatinib-resistant mutations, except for T315I. [159,160]. Other nilotinib-resistant mutations seen in patients are E255K/V, Y253H, and F359V/C/I [139]. The kinase inhibition spectrum is much narrower compared to

dasatinib (Fig. 8) and the main targets, in addition to BCR-ABL1, are C-KIT and PDGF receptors [124]. Nilotinib is an effective treatment for CML with imatinib resistance and tolerance, [161-163] although in imatinib-resistant Ph+ ALL patients, nilotinib showed only limited efficacy. [161] Patients with nilotinib-resistant mutations had less favorable responses to nilotinib. [164] A phase 3 study, called Evaluating Nilotinib Efficacy and Safety in Clinical Trials–Newly Diagnoses Patients (ENESTnd), compared imatinib and nilotinib in newly diagnosed CML patients. As in the case of dasatinib, responses with nilotinib were faster and deeper when compared to imatinib responses. [165,166] Also nilotinib has been approved for the treatment of newly diagnosed CML patients.

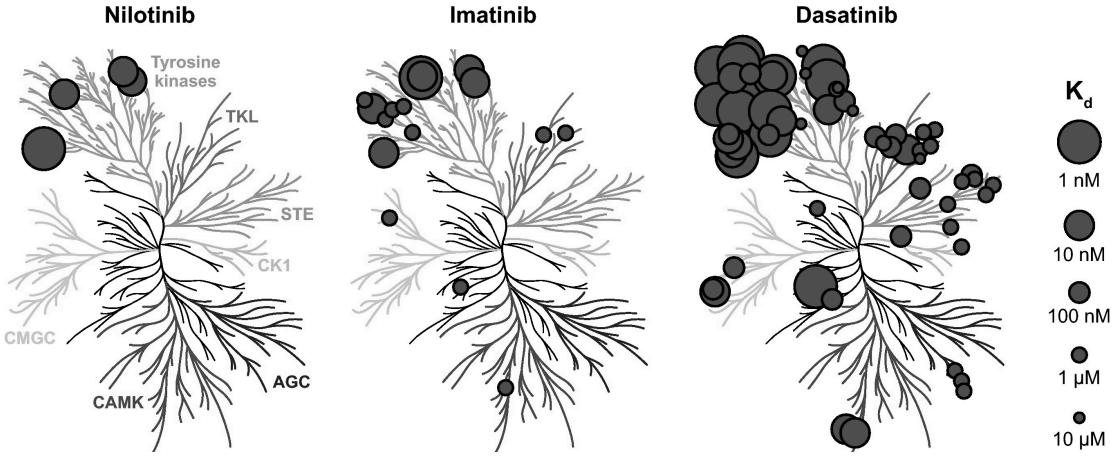

Figure 8. *Comparison of the kinase inhibition spectrum and the affinity of nilotinib, imatinib, and dasatinib throughout the kinome. Kd (dissociation constant) describing the affinity of inhibitor to kinase. Reprinted with permission. © 2012 Elsevier.* [167]

2.2.6.6 Other tyrosine kinase inhibitors that target BCR-ABL1

Bosutinib (previously SKI-606) is a second-generation TKI with a target spectrum consisting of BCR-ABL1, SRC, TEC, and STE20 family kinases. In contrast to imatinib, dasatinib, and nilotinib, bosutinib does not inhibit C-KIT and PDGF receptors. However, as seen with other TKIs, the T315I and V299L mutations were inaccessible with bosutinib. [168,169] Bosutinib demonstrated efficacy with imatinib-resistant and tolerant CML patients. [170] Patients who had failed either with dasatinib or nilotinib, or both, also benefited from bosutinib therapy. Bosutinib therapy induced responses with patients carrying dasatinib-resistant F317L mutation and nilotinib-resistant F359C/I/V mutations. [171] Bosutinib was recently approved for the treatment of CML patients with tolerance to other therapies.

Bafetinib (INNO-406) is a BCR-ABL1 inhibitor with specific LYN (SRC family kinase) targeting properties, but this inhibitor has no efficacy against the T315I mutation [172]. Clinical trials with imatinib resistant and intolerant CML and Ph+ ALL patients are ongoing. Bafetinib showed efficacy with patients resistant to multiple TKIs in a phase 1 study, although further studies are required to evaluate its efficacy. [173]

Treatment options for patients with resistance to imatinib and second-generation TKIs are scarce. In the case of the T315I mutation and advanced phase CML, allogeneic stem cell transplantation is recommended. [174] IFN-α therapy has been effective in combination with imatinib and new studies evaluating IFN-α as combination with dasatinib and nilotinib are about to start. [175] In some rare cases, IFN-α has been able to induce responses in combination with TKIs in patients carrying the T315I mutation. [176,177]

Third generation TKIs are emerging in the field of CML therapy, to overcome the resistance seen with previous inhibitors. In their development, emphasis has been on the T315I mutation and several candidates are on preclinical and phase 1 trials. [174] One of the most promising TKIs able to inhibit the T315I mutation is ponatinib (AP24534). In addition, Ponatinib inhibits SRC, VEGFR, FGFR, and PDGFR family kinases. [178] Clinical trials with ponatinib are ongoing and initial results from a phase 2 trial, called The Ponatinib Ph+ ALL and CML Evaluation (PACE), involves CML and Ph+ ALL patients with dasatinib and/or nilotinib intolerance or resistance (including T315I). Results are promising: after a 6 month follow-up, 38/61 of chronic phase CML patients with the T315I mutation had entered major CyR.[179]

2.2.6.7 Mechanisms of imatinib resistance in CML

Mechanisms behind imatinib resistance have been well defined. The major reason for resistance is BCR-ABL1 dependent, but mechanisms independent from BCR-ABL1 have also been defined. In patients, the main reason for imatinib resistance are point mutations in the *BCR-ABL1* genes, which result in amino acid changes in the drug-binding site; over 90 different imatinib-resistant mutations have been defined in patients. [139] These mutations are present in different parts of the KD: the activation loop, the phosphate binding P-loop, and the hinge region. [180] The imatinib-resistant mutated clones probably pre-exist at minute levels before start of imatinib-therapy, but expand during the course of the treatment as result of selective pressure. [181] Imatinib-resistant mutations, including T315I, have been observed previously before therapy administration and at the time of diagnosis [182,183]

A characteristic of imatinib-resistant mutations is a shift to a more active conformation, because of destabilization of the P-loop and the DFG motif. This conformational change increases the free energy needed for imatinib binding and mutations in the P-loop are responsible for this kind of destabilization of inactive conformation. The T315I mutation is called the gatekeeper mutation in the hinge region as it is responsible for disrupting a key H-bond with imatinib. The substitution of threonine with isoleucine causes steric hindrance and the loss of hydrogen bond affinity with imatinib. [184] Nilotinib, similar to imatinib, is specific for the inactive

conformation of the KD. The nilotinib mechanism is efficient at inhibiting the imatinib-resistant mutations, most likely due to high affinity BCR-ABL1, which shifts the equilibrium towards the inactive state. [184] Dasatinib is able to bind both inactive and active states of the protein, and thus the binding properties are less stringent than for imatinib and nilotinib. This enables binding to many imatinib-resistant mutations and is also the basis for specificity for the SRC family kinases, since the active conformation of BCR-ABL1 and the SRC family KDs share structural similarities. [185] Ponatinib is specific for the inactive DFG-out conformation, but also possesses a unique ethinyl linker, which is able to skirt the effects of the bulky isoleucine in the T315I mutation. [186]

A second BCR-ABL1 dependent mechanism of resistance is amplification of the *BCR-ABL1* fusion gene, which has been reported in imatinib-resistant cell lines. [187] This phenomenon is rarely seen in patients, since *BCR-ABL1* gene amplification was only detectable in 2 out of 32 imatinib-resistant CML patients analyzed with cytogenetics [140]. On the other hand, amplification of *BCR-ABL1* might play a role in the primitive CD34+ cells, since protein expression was higher in these cells in patients with blast crisis than in patients in the chronic phase. [188]

Several BCR-ABL1 independent mechanisms have been also proposed. Differences in pharmacokinetics of imatinib, mediated by cytochrome P450 protein levels, can affect the imatinib plasma levels, [189] which in turn is associated with therapy responses. [190] Intracellular uptake of imatinib is mediated by different influx and efflux mechanisms. Regulation of imatinib influx by the human organic cation transporter (hOCT1) has been proposed to have an impact on cellular imatinib levels. [191] Imatinib is also a substrate for the efflux protein MDR1 (Multi drug resistance protein 1, also known as ABCB1), a transporter associated with multidrug resistance. [192] MDR1 is overexpressed in blast phase CML patients and is implicated in imatinib resistance. [187]

One theory for resistance is stem cell persistence against imatinib therapy; these cells then serve as a reservoir for the malignancy. [193] Different mechanisms behind stem cell resistance have been postulated; for example, reduced imatinib exposure in the BM niche or altered drug intake and efflux. [194] Primitive imatinib-resistant cells also possess higher amounts of *BCR-ABL1* transcripts and protein compared to mature cells. [195] CML stem cells are also insensitive to other TKIs, such as dasatinib, nilotinib, and bosutinib. [195-197]

SRC family kinases have been implicated in the pathogenesis of CML, [198,199] while some evidence claims a more important role for Ph+ ALL. [200] High expression of HCK and LYN has been reported to take part disease progression and imatinib-resistance. [201-204] TKIs, like dasatinib, that are able to inhibit SRC family kinases have the possibility to overcome resistance caused by overactive SRC signaling. Bosutinib, in particular, which is a specific inhibitor of LYN, has been implicated in causing superior responses compared to imatinib through LYN inhibition.

2.2.7 Monitoring of therapy response in CML

2.2.7.1 Genetic techniques

Several genetic techniques are utilized in the process of diagnosing of CML, which are also useful for monitoring the therapy response and evaluating prognosis.

Diagnosis and follow-up of CML is based on presence of the Ph chromosome and the *BCR-ABL1* fusion gene. At diagnosis, a sample can be either PB or BM, but once patient has reached a hematologic response (HR), BM as source of sampling for cytogenetic analysis is recommended. At diagnosis, the presence of the Ph chromosome is often noticed in G-banding analysis of metaphasic cells. Karyotyping with G-banding is a conventional cytogenetic technique that is able to detect abnormalities in chromosomes, such as deletions, inversions, insertions, and translocations in dividing cells.[205] [206]

At diagnosis, other chromosomal abnormalities are detectable, which are associated with the accelerated phase. A closer cytogenetic analysis is performed using *BCR-ABL1* specific fluorescence *in situ* hybridization (FISH) with 5´BCR and a 3´ABL1 fluorescent probe with different colors. The FISH technique is based on fluorescently labeled probe hybridization into metaphase chromosomes, interphase nuclei, or extended chromatin fibers. FISH can be performed with either metaphase or interphase cells. [207]

After the introduction of TKIs as a therapy for CML and a high level of complete cytogenetic responses, the demand has increased for higher sensitivity analysis than FISH. The real-time quantitative polymerase chain reaction (RQ-PCR) is by far the most sensitive and accurate method and it is especially useful in monitoring minimal residual disease (MRD) from deoxyribonucleic acid (DNA), complementary deoxyribonucleic acid (cDNA), or ribonucleic acid (RNA) samples. RQ-PCR is a quantitative PCR method based on specific forward and reverse primers and measurable fluorescent dye. The amount of PCR product is determined by the number of cycles required to reach a certain threshold level in a proportional standard sample. [208] RQ-PCR is now routinely used to monitor responses to TKI therapy in CML patients from PB samples. [207] The level of *BCR-ABL1* transcript is determined in relation to reference genes such as *GUS, ABL1*, or *BCR*. The control gene has been chosen to meet certain criteria: the expression level should be similar to *BCR-ABL1* at diagnosis, the mRNA stability should be similar to *BCR-ABL1*, and the primers should not amplify genomic DNA sequences. [209] Different break variants, e13a2 (b2a2) or e14a2 (b3a2), are determined individually for each patient at the time diagnosis and the same variant is used during the follow-up.

In HR, the blood leukocyte and platelet counts are normalized and spleen reverts to normal size, but these are not informative regarding the numbers of leukemic cells. The cytogenetic response is determined by the number of cells carrying the Ph chromosome, determined by conventional cytogenetics, and undetectable Ph positive cells in BM constitute CCyR. Patients with CCyR may still carry up to 10^9 leukemic cells, which can be detected by RQ-PCR. The major molecular response (MMR) is defined by ≥3-log reduction of the *BCR-ABL1* transcript to the reference gene. The patient is regarded as being in complete molecular response (CMR) when no *BCR-*

ABL1 transcripts are detectable. Different responses are listed in Table 2. [210] The present way of reporting results is being replaced by a new international scale (IS) standard, because of the unreliable results seen with the log-system. The IS system aims to provide a more harmonized method between diagnostic laboratories. A score of 100% in the IS system is given to the baseline level of *BCR-ABL1* in newly diagnosed patients, and a score of 0.1% indicates MMR (Table 2). [209] However, even deeper responses can be measured currently, referred to as MR4.0 (≥4 -log reduction), MR4.5, and MR5.0, which are comparable with 0.01%, 0.0032%, and 0.001%, respectively, on the IS-scale. [211]

***Table 2**. Responses to TKI therapy defined by BCR-ABL1 ratio and leukemic cell numbera, and the IS standard. Modified from Vigil et al. 2011.* [210]

Leukemic cells	Response	IS standard score (%)
10^{12}	-	100
10^{10}	CHR	10
10^{9}	CCyR	1
10^{6}	MMR	0.1
	CMR	Undetectable

2.2.7.2 FACS techniques

Fluorescence-activated cell sorting (FACS) can be utilized in the analysis of heterogeneous cell populations. The technique is based on the light scattering properties of cells: when labeled with fluorescently tagged chemicals, cells emit fluorescent light when excited with lasers. By the use of different cell surface markers, analysis of various cell populations can be conducted.[212] In addition to phenotypic analysis, FACS can be used in functional studies measuring cytokine production[213], cytotoxicity[214] and apoptosis[215]. With the growing availability of conjugated monoclonal antibodies, sophisticated instrumentation and improved software, an increased amount of parameters can be analyzed using FACS. Currently 19-parameter analysis is possible, although this is not a reality in clinical settings.[216]

FACS is an essential tool in the analysis of leukemic clones, especially in ALL and acute myeloid leukemia (AML). [217,218] The use of FACS analysis is not commonly used in CML diagnosis and MRD analysis, as monitoring TKI-response with PCR methods is highly sensitive. A leukemia-associated phenotype is also not present in CML. However, CML progression is usually associated with clonal evolution for which FACS analysis is useful in determining the characteristics of the disease.[219]

CML can also be monitored though intracellular staining. The phosphorylation of the CRKL protein downstream of BCR-ABL1 has been shown to indicate the presence and activity of the oncogenic fusion protein, however this method has not yet been adapted to routine clinical use.[220] FACS bead assay has been developed for the detection of leukemic fusion proteins from lysed leukemia samples. Assay specific for analyzing the BCR-ABL1 fusion protein from CML patient samples has a bead-bound

catching antibody specific for the BCR-part and a fluorochrome-conjugated detection antibody against the ABL1 part. The method was validated using BCR-ABL1 positive cell lines from CML and ALL patient samples. The sensitivity of the assay was between 0.1%-1%, indicating higher sensitivity than FISH or karyotyping, but less than quantitative PCR. Results with patient samples were concordant to those conducted with PCR, although sensitivity of the assay may be problematic in the assessment of MRD. Benefits over traditional PCR and cytogenetic techniques are faster processing time and freedom from specialized laboratory facilities. Specific analyses of the different BCR-ABL1 variants are not needed, since the assay is specific for all of them. Fusion protein analysis can also be combined simultaneously with other FACS analyses. Due to these benefits FACS analysis of BCR-ABL1 protein has recently entered routine clinical use.[221]

2.2.8 Prognostic factors for therapy outcomes in CML

2.2.8.1 Factors based on clinical features

Prognostic scoring systems predicting therapy responses have existed since before the imatinib era. One widely used system is the Sokal score, derived from CML patients using mainly busulfan. This factor is based on diagnostic phase age, spleen size, platelet count, and PB blasts and it separates patients into low, intermediate, and high-risk groups by predicting 4-year survival probabilities. [222] The Sokal score was insufficient at discriminating between low and intermediate risk groups in IFN-α-treated patients. Therefore, a new prognostic factor was derived, the Hasford score, which also includes blood eosinophils and basophils in the variables. [223] Deletions at the 9;22 breakpoint, [224] and a lack of certain regions in the chromosome 9, also implicated poor prognosis for patients treated with IFN-α and hydroxyurea. [225] After introduction of imatinib as the treatment for CML, the efficacy of these parameters has shown less potential. For example, deletions in chromosome 9 no longer influence the survival of imatinib-treated patients [226] or patients treated with second generation TKIs. [227] The IRIS study concluded that Sokal scores given to patients at their diagnostic phases correlated with their rates of cytogenetic responses with imatinib treatment, with the lowest rate occurring in the high-risk group. The risk for disease progression was also significantly higher for the high-risk group. [228] However, the Sokal score did not correlate with disease progression after achieving a CCyR among imatinib-treated patients [228], while the Sokal score was an important predictor of outcome after achieving CCyR with IFN-α -treated patients. [229] European LeukemiaNet has developed new scoring system EUTOS (European Treatment and Outcome Study) on the basis of newly diagnosed CML patients treated with imatinib-based regimens. This factor is based on the percentage of basophils and spleen size and divides patients into low and high-risk groups. Validation of the EUTOS score stated that 34% of CML patients failed to achieve CCyR in 18 months in the high-risk group. [230] The effectiveness of EUTOS score was questioned in a recent study, as it was unable to predict outcome of CML patients treated with imatinib or second generation TKIs. [231]

The response time is also connected to the imatinib therapy outcome[133,228]. The achievement of MMR within a 12-month time frame is associated with a longer duration of CCyR in imatinib-treated patients and considered a predictive factor for a

good clinical outcome. [232] Failure to achieve MMR within 12 months is associated as a risk factor, while failure within 18 months is considered a suboptimal response.[233] Similar predictive values (MMR, MCyR) have also been demonstrated with second-generation TKIs after imatinib-failure. [234,235] A recent publication demonstrates that the measurement of *BCR-ABL1* transcripts in accordance to the IS scale can predict a clinical outcome for TKIs in as early as 3 months also. [236,237]

2.2.8.2 Predictive genetic factors

As the traditional parameters have somewhat limited efficiency, a demand has grown for new prognostic factors such as biomarkers. Genetic approaches have discovered differentially expressed genes in cells from primary patient with different disease stages. [238,239] Multiple studies have identified a set of differentially expressed genes according to the imatinib response. [240-244] However, the results have not clearly indicated the genes connected to the imatinib response and one of these studies was unable to predict a cytogenetic response to imatinib. [245]

Different kinds of genetic polymorphisms have also been identified in association with the imatinib response. These genes are usually associated with drug absorption, distribution, metabolism, and excretion. This is likely connected to the fact that higher imatinib plasma levels are associated with better responses [190]. Different polymorphisms, such as T1236C, G2677T/A, and C3435T, in the *ABCB1* gene that encodes the MDR1 efflux protein are associated with certain imatinib responses. [246] The same kind of association has been discovered for the polymorphic variants C421A and G34A of the *ABCG2* gene that encodes another imatinib efflux protein, the breast cancer resistance protein (BCRP). [247] Low hOCT1 protein activity is associated with lower probability of MMR, [248] and consequently high expression of this imatinib influx protein is connected with better survival and responses, while a similar connection with the previously mentioned efflux proteins was absent. [249] The C480G and A1222G polymorphisms in the hOCT1-encoding *SLC22A1* gene also show some predictive characteristics. [247,250] Finally, the *CYP3A5* gene responsible encoding of the imatinib-metabolizing enzyme, P450, predicts a response for imatinib through polymorphism at A6986G. [250] In general, the results from different studies are conflicting, depending on the ethnicity of the patients, group size, drug dosage, and study design, as concluded in a review article by Dulucq and Krajinovic. [251] These findings diminish the usability of these genetic polymorphisms as biomarkers for the imatinib response.

Germ line polymorphism in the *BIM* gene encoding for BCL2-like (BIM) protein has been linked to imatinib. Deletion in intron 2 was noticed to favor splicing of exon 3 over exon 4 of the *BIM* gene. Expression of exon 3 resulted in expression of a BIM isoform that lacked the pro-apoptotic BCL2-homology domain (BH3) that causes imatinib resistance in CML and EGFR-mutated NSCLC cell lines. Resistance to imatinib was overcome with BH3 mimetic drugs. This polymorphism in the *BIM* gene was also more common in East Asian population (12.3 %), while absent in European and African individuals. The *BIM* deletion polymorphism was associated with clinical resistance in CML patients treated imatinib, since patients with the polymorphism causing this deletion had poorer responses to imatinib compared to patients without the polymorphism. [252] In East Asia, the frequency (~50%) of incomplete cytogenetic

responses is higher among imatinib-treated CML patients [253] when compared to European and North American patients (26 %), [134] which can be partly explained by a different genetic background. Authors estimate that *BIM* polymorphism might be responsible for 21% of the imatinib resistance seen in East Asia and suggest it as a germline biomarker for TKI resistance, as it is easily testable during diagnosis. [252]

In addition, CML patients with a killer immunoglobulin-like receptor (KIR) 2DS1 genotype predicted a failure to achieve CCyR and inferior progression free survival and overall survival. [254] A specific IFN-γ genotype was also connected with CCyR and MMR in Canadian and Korean patients, suggesting a role for IFN-γ signaling in CML pathogenesis. [255]

3 AIMS OF THE STUDY

The overall aim of the study was to characterize aberrant cell signaling patterns in CML cells and in normal leukocytes during TKI therapy and to discover novel biomarkers for therapy response. In addition, we aimed to understand the molecular basis of TKI response in a Ph negative ALL patient.

Specific aims of the subprojects included:

1) To investigate the molecular mechanism underlying ABL1-driven ALL

2) To characterize the effects of TKI therapy on immune cell function in CML and associate the signaling pattern with therapy responses

3) To develop a novel automated method for FACS data analysis

4) To discover novel biomarkers for the imatinib response in CML patients

4 PATIENTS AND METHODS

4.1 Patients and ethical permissions

The patient in Study I (patient #1, Table 3) had a pre-B ALL. The patient had a treatment history of dasatinib combined with chemotherapy, prior to allogeneic HSCT. The patient relapsed after the transplantation and continued with dasatinib therapy. Sample for Study I was obtained during the diagnosis.

Study II consisted of 10 CML patients in the diagnostic phase and 10 imatinib-treated and 10 dasatinib-treated CML patients (Table 3), all in chronic phase. Most of the patients had a CCyR, while one imatinib-treated patient (patient #3) had partial cytogenetic response with 25 % Ph + cells in the BM; one dasatinib-treated patient was only in HR (patient #18). Most of the dasatinib-treated patients had been treated with imatinib prior to dasatinib therapy due to inadequate responses (excluding patients #8 and #11). Four patients (patients #16, #19, #21, and #24) had also been treated with IFN-α before imatinib. The same patient data were used in Study III.

Study IV included 10 diagnostic phase CML patients, who achieved either MMR or CMR for imatinib at 18 months. These patients were designated as an optimal response group. The suboptimal response group, with 10 diagnostic phase patients, achieved CCyR, but no MMR after 18 months of imatinib therapy. Patients #27, #28, #30, and #31 also received IFN-α after 3 months imatinib monotherapy. The standard 400 mg dose was increased to 600-800 mg after 3-12 months of therapy due to suboptimal response with 5 patients (#13, #33, #35, #36, and #37). This study also included patients who had failed with TKI therapy. One chronic phase CML patient (#39) achieved only HR for imatinib and carried an imatinib-resistant M351T mutation. This patient did not achieve a cytogenetic response to either bosutinib or nilotinib. Chronic phase patient #40 achieved a minimal cytogenetic response (MinCyR) for imatinib and nilotinib and patient #41 was in blast crisis during BM sampling after a short period of imatinib therapy.

Study II included 7 healthy controls for blood samples and Study IV included 4 healthy controls for BM samples; these persons had median ages of 25 and 29 years, respectively, and normal blood counts.

The study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Helsinki University Central Hospital Ethics Committee. Written informed consent was obtained from all patients and healthy controls.

***Table 3**. Patient characteristics*

Pt #	Malignancy	Age	Gender	Treatment	Dosage/ mg	Sokal group (CML)	Response at sampling/mo (study II)	Imatinib treatment response 18 mo (study IV)	Study
1	B-ALL	40	Male	Dasatinib*	140	n/a	n/a	n/a	I
2	CML	43	Male	Dg/Imatinib	400	LR	CCyR/6	n/a	II,III
3	CML	73	Male	Dg/Imatinib	400	HR	PCyR/5	n/a	II,III
4	CML	49	Male	Dg	n/a	LR	n/a	n/a	II,III
5	CML	63	Female	Dg/Imatinib	400	IR	n/a	CMR	II,III,IV
6	CML	34	Female	Dg/Imatinib	400	LR	CCyR/5	CMR	II,III,IV
7	CML	30	Male	Dg/Imatinib	400	LR	n/a	MMR	II,III,IV
8	CML	45	Male	Dg/Dasatinib	100	LR	CCyR/6	n/a	II,III
9	CML	55	Female	Imatinib	400	IR	CCyR/21	n/a	II,III
10	CML	62	Male	Dg/Imatinib	400	LR	n/a	MMR	II,III,IV
11	CML	60	Female	Dg/Dasatinib	100	IR	CCyR/6	n/a	II,III
12	CML	55	Female	Dg/Imatinib	400	IR	CCyR/6	n/a	II,III
13	CML	41	Male	Imatinib	400	IR	CCyR/31	CCyR	II,III,IV
14	CML	61	Male	Imatinib	400	LR	CCyR/21	CCyR	II,III,IV
15	CML	53	Male	Imatinib	400	IR	CCyR/19	CCyR	II,III,IV
16	CML	57	Male	Imatinib	400	IR	CCyR/31	n/a	II,III
17	CML	60	Male	Imatinib	400	LR	CCyR/26	n/a	II,III
18	CML	73	Male	Dasatinib	100	HR	CHR/1	n/a	II,III
19	CML	30	Male	Dasatinib	100	LR	CCyR/16	n/a	II,III
20	CML	65	Female	Dasatinib	100	IR	CCyR/16	n/a	II,III
21	CML	53	Male	Dasatinib	140	LR	CCyR/37	n/a	II,III
22	CML	28	Female	Dasatinib	140	HR	CCyR/37	n/a	II,III
23	CML	64	Male	Dasatinib	100	HR	CCyR/20	n/a	II,III
24	CML	42	Male	Dasatinib	100	HR	CCyR/35	n/a	II,III
25	CML	68	Male	Dasatinib	100	LR	CCyR/3	n/a	II,III
26	CML	49	Male	Imatinib	400	LR	n/a	CMR	IV
27	CML	48	Male	Imatinib	400	IR	n/a	CMR	IV
28	CML	38	Female	Imatinib	400	LR	n/a	CMR	IV
29	CML	51	Female	Imatinib	400	LR	n/a	CMR	IV
30	CML	55	Male	Imatinib	400	LR	n/a	CMR	IV
31	CML	72	Male	Imatinib	400	IR	n/a	CMR	IV
32	CML	43	Male	Imatinib	400	LR	n/a	CCyR	IV
33	CML	35	Male	Imatinib	400	LR	n/a	CCyR	IV
34	CML	67	Male	Imatinib	400	LR	n/a	CCyR	IV
35	CML	27	Male	Imatinib	400	LR	n/a	CCyR	IV
36	CML	54	Male	Imatinib	400	IR	n/a	CCyR	IV
37	CML	62	Female	Imatinib	400	IR	n/a	CCyR	IV
38	CML	40	Male	Imatinib	400	IR	n/a	CCyR	IV
39	CML	51	Female	**	n/a	HR	n/a	CHR	IV
40	CML	69	Male	***	n/a	HR	n/a	MinCyR	IV
41	CML	35	Male	Imatinib	n/a	HR	n/a	BC	IV

** dasatinib in combination with chemotherapy, ** therapy history with imatinib, bosutinib and dasatinib, *** therapy history with imatinib and nilotinib, BC=blast crisis; LR, low risk; IR, intermediate risk; HR; high risk.*

4.2 Methods

4.2.1 Mononuclear cell isolation from blood and bone marrow (BM) and storage (I, IV)

Mononuclear cells were isolated from blood (Study I) and BM (Study IV) with the Ficoll-Paque system (GE Healthcare, Piscataway, NJ, USA), according to the manufacturer´s instructions. Cells were washed once with phosphate buffered saline (PBS) and counted before snap freezing as pellets at -70 ^{o}C. Portions of the BM samples were cryopreserved in fetal bovine serum (FBS, Lonza, Basel, Switzerland) and 10 % dimethyl sulfoxide (DMSO).

4.2.2 Cell culture (IV)

K562 cells (Sigma Aldrich, St. Louis, MO, US) and EM-2 and MOLM-1 cell lines (both DSMZ, Braunschweig, Germany) were grown in RPMI 1640 media containing 10% FBS (20% for MOLM-1), 2mM L-glutamine, 1% penicillin and 1% streptomycin (all Lonza). Cells were washed once with PBS before freezing at -70 ^{o}C as snap-frozen pellets.

4.2.3 Cytogenetic characterization of the t(1;9)(q24;q34) translocation (I)

Patient #1 was analyzed with a routine G-banding method during diagnosis. More detailed analysis was performed with multicolor FISH (Metasystems GmbH, Altlussheim, Germany), chromosome 9 painting and FISH primers designed to bind 1p36 and 1q25 regions of chromosome 1. The patient was also analyzed using a *BCR-ABL1* fusion gene with a *BCR-ABL1* ES dual color extra signal translocation probe (Vysis®, Abbott Laboratories, IL, USA) and PCR analysis.

4.2.4 PCR reaction and DNA sequencing of the *RCSD1-ABL1* fusion gene (I)

RNA and cDNA were generated by standard molecular biology methods from a pre-B lymphoblastic leukemia patient's sample (patient #1). Forward PCR primers were designed to anneal with the *RCSD1* gene and reverse primers with the *ABL1* gene (Table 4, Fig. 9). Reverse primers were designed to overlap with the ABL1 KD, which is situated in the exons 5-7 of the *ABL1* gene. The PCR reactions were performed with all six possible primer combinations with 0.5 μM concentrations each. Other reagents of the PCR reaction included: Phusion polymerase (1U), GC buffer (1x), dNTP mix (200 μM) (all Finnzymes, Espoo, Finland) and 125 ng template DNA (patient cDNA or control cDNA) or water as a control. The PCR program consisted of initial denaturation (30s/98^{o}C), 30 cycles of denaturation (10s/98 ^{o}C), + annealing (30s/62 ^{o}C), + extension (10min/72 ^{o}C), and a final extension (10min/72 ^{o}C).

PCR products were analyzed with 1% and 2% agarose gels with a 1 kb DNA ladder (New England Biolabs, Ipswich, MA, USA). PCR products were purified with a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) from the 2 % agarose gel and sequenced with their respective primers.

Table 4. *Primers designed for analysis of the RCSD1-ABL1 fusion gene.*

Primer	Sequence	Gene	Exon
RCSD1-aF	5'-CCTGAAGGACATGGAGGAAA-3'	*RCSD1*	1-2
RCSD1-bF	5'-CAGAGACCAATGCCAATGTG-3'	*RCSD1*	2
ABL1-aR	5'-CTGCACCAGGTTAGGGTGTT-3'	*ABL1*	6
ABL1-bR	5'-TGGTGTCCTCCTTCAAGGTC-3'	*ABL1*	5-6
ABL1-cR	5'- TCTGAGTGGCCATGTACAGC-3`	*ABL1*	7

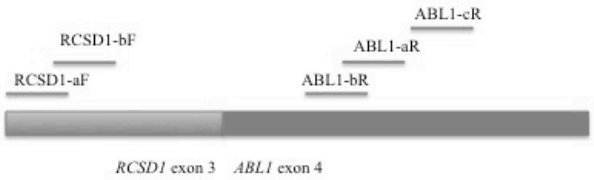

Figure 9. *RCSD1-ABL1 fusion gene and annealing sites of PCR primers.*

4.2.5 Single cell phosphoprotein analysis (II)

Principle of single cell phosphoprotein analysis was to measure cytokine-induced phosphoprotein signals in individual leukocyte subtypes. The signaling proteins measured with this method were extracellular-signal-regulated kinase 1/2 (ERK1/2) and proteins belonging to the signal transducer and activator of transcription (STAT) family.

ERK1/2 belongs to the MAPK family. ERK1/2 is involved in many cellular processes and is inducible with various mitogens, growth factors, and cytokines. [256] Activation STAT proteins are mediated with wide array of cytokines and growth factors. Binding of the extracellular ligand to its specific receptor results phosphorylation of Janus kinase protein (JAK) via the cytoplasmic part of the receptor. Activated of JAKs phosphorylate STAT proteins, which dimerize and enter the nucleus, where they take action as transcription factors. The JAK-STAT pathway is essential in mediating cell proliferation, differentiation, cell migration and apoptosis, but is also associated with different types of cancer. [257]

4.2.5.1 Ex vivo cytokine cell activation

Freshly drawn blood in heparin tubes from healthy controls, diagnostic CML patients, and CML patients receiving imatinib and dasatinib (Table 3) were stored at +37 ^{o}C for a maximum of 1 hour before cytokine activation. Aliquoted samples were activated (15 min, +37 ^{o}C) with GM-CSF (100 ng/ml), a mixture of IL-2 (100 ng/ml), IL-10 (100 ng/ml), and IFN-α; 100 U/ml) and a mixture of IL-4 (100 ng/mL), IL-6 (100 ng/ml), and IFN-γ (100 ng/ml). The manufacturer of IFN-α was PBL Interferon Source (Piscataway, NJ, USA) and GM-CSF, IFN-γ, IL-2, IL-4, IL-6, and IL-10 were

obtained from R&D Systems (Minneapolis, MN, USA). PBS stimulated blood was used as stimulation control and aliquots of blood without PBS or cytokine stimulation were used to determine baseline phosphoprotein analysis. Red blood cells were lysed with (10 min, RT) FACS lysing solution (BD Biosciences, San Jose, CA, USA) and samples were fixed with paraformaldehyde in PBS, with final concentration of 2 %. Cells were washed once with PBS to remove residual lysed red blood cells and stored in -70 ^{o}C).

4.2.5.2 Phosphoprotein staining

Antibodies used for the phosphoprotein flow cytometry were CD45 APC-H7, CD4 PerCP, CD3 PE-Cy7, CD25 PE-Cy7, STAT3 A647 (Y705), STAT5a-A488 (Y694), STAT6 PE (Y641), ERK1/2 A488 (T202/Y204), STAT1 PE (Y701), and STAT1 A488 (Y701) (BD Biosciences) (Table 5). Suitability of detecting the surface markers with the permeabilization step was first tested and optimal antibody concentrations were determined. Samples at -70 ^{o}C were thawed and washed with PBS. Cells were stained with CD45 APC-H7 (RT, 15 min), washed once with PBS, and permeabilized for 10 min on ice with -20 ^{o}C methanol. After removing the residual methanol with two PBS washes, the cells were stained with the remaining antibodies according to the individual antibody panel. Panels used in the staining were PBS-stimulated cells: panels 1–4; GM-CSF –stimulated cells: panels 1–2; IL-2, IL-10, IFN-α -stimulated cells: panels 1 and 3; of IL-4, IL-6, IFN-γ -stimulated cells: panels 1 and 4. After one hour of incubation on ice, the cells were washed and analyzed with a six-color flow cytometer (FACS CantoI or CantoII, BD Biosciences).

Table 5. *Antibody panels used in multicolor FACS analysis*

	A488	PE	PerCP	PE-Cy7	A647	APC-H7
1	ERK1/2	STAT1	CD4	CD3	STAT3	CD45
2	STAT5	STAT1	CD4	CD3	STAT3	CD45
3	STAT5	STAT1	CD4	CD25	STAT3	CD45
4	STAT1	STAT6	CD4	CD25	STAT3	CD45

4.2.5.3 Phosphoprotein data analysis

Cell gating was performed with open access Cytobank software (http://www.cytobank.org/) and FACS Diva software (Version 6.1.2; BD Biosciences). Granulocyte, lymphocyte, and monocyte populations were gated from side scatter (SSC) versus CD45. Lymphocytes from this scatter were further gated according to CD4 expression, and regulatory T cells (Treg) were analyzed from CD4- and CD25 positive cells. Intracellular protein intensities STAT1, STAT3, STAT5, STAT6 and ERK1/2 were measured by subtracting the PBS-activated signal from the cytokine-activated signal. Baseline phosphoproteins were analyzed from samples without any activation (PBS or cytokine). Data were analyzed by comparing the different study groups with GraphPad (Version 5.2) software using the Kruskal-Wallis test and Dunn's Multiple Comparison test. The limit for a statistically significant result was set as $p<0.05$.

4.2.6 IL-2, IL-4, IL-6, IL-10, IFN-α, IFN-γ, and GM-CSF concentration measurements (II)

Plasma samples from healthy controls, diagnostic CML patients, and patients treated with imatinib and dasatinib were analyzed with a Human Cytokine 25-plex Panel (Invitrogen, Camarillo, CA, USA) according to the manufacturer's instructions. Measurement and analysis were performed using a Bio-Plex 200 System (BioRad, Hercules, CA, USA). The measurement was conducted for corresponding time points as the cytokine activations were performed.

4.2.7 Automated FACS data analysis (III)

The FlowAnd FACS data analysis tools were implemented into the freely available Anduril framework developed by Ovaska et al. [258]. Five different modules were built into the FlowAnd, including: data import, preprocessing, gating, population identification, and data analysis. The majority of these modules had been developed as part of previously developed software. The data import part was from flowCore [259] and the gating module utilized three different clustering methods: SamSPECTRAL [260], flowMEANS [261], and the mixture modeling from FLAME software [262]. The clustering is associated with a graphical module, where the user can identify the clusters. FACS data from project II were utilized in this project to validate the reproducibility and feasibility of FlowAnd compared to manual analysis.

4.2.8 Kinase target activation profiling (IV)

4.2.8.1 Lysate preparation and kinase array

The Proteome Profiler Human Phospho-Kinase Array Kit (R&D systems, Minneapolis, MN, USA) was used to analyze phosphorylation of 46 different kinase target sites (http://www.rndsystems.com/product_results.aspx?k=ary003). Diagnostic phase CML patient's BM mononuclear cells (MNCs), with optimal and suboptimal imatinib responses, healthy control BM MNCs, K562, EM-2 and MOLM-1 cells were stored snap-frozen and thawed before analysis. Three additional patients, without

appropriate therapy response to various TKIs, were also analyzed (Patients #39, #40 and #41, Table 3). Cells were lysed according to the manufacturer's directions (with lysis buffer from the Kinase Array Kit). In addition, Mini EDTA-free protease inhibitors (Roche Diagnostic, Mannheim, Germany) were used in the lysis buffer as a 7x concentrate in PBS. Protein concentration was measured with a Qubit Fluorometer (Invitrogen, Carlsbad, CA, USA) and 300 μg protein was used in each experiment. Signal detection for the array was performed with Super Signal West Femto Maximum Sensitivity ECL reagents (Thermo Scientific, IL, USA). The x-ray films were exposed to the array membranes for several different exposure times.

4.2.8.2 Data analysis

X-ray images were scanned and intensities of each spot were analyzed by custom code in the Anduril analysis software [258]. Spot intensities were analyzed by subtracting the background intensity obtained from negative PBS controls and the mean value was determined between duplicates. Optimal and suboptimal groups were compared with a non-parametric Mann Whitney test. Other statistical comparisons performed were healthy controls vs. CML patients and CML patients divided into low risk and intermediate Sokal risk factor groups.

5 RESULTS

5.1 1;9 translocation (I)

In order to understand the molecular mechanism of dasatinib sensitivity in an Ph neg ALL patient (patient #1), the diagnostic phase leukemic cells with cytogenetic and molecular methods was investigated.

5.1.1 Clinical characterization of the patient

The patient had been diagnosed with CD19+, CD22+, CD10+, cCD79a+, nTdT+, CD34+, CD38+, and HLA-DR+ B-ALL in March 2007 and BM aspirate showed 80% of blast cells. The patient received two courses of cyclophosphamide, vincristine, doxorubicin (Adriamycin®) and dexamethasone (CVAD) as an induction therapy, but leukemia was chemotherapy resistant. FISH analysis after the 2 courses of CVAD revealed that still 50 % of blasts were in the BM. Based on the diagnostic phase cytogenetic results showing t(1;9) translocation, dasatinib (140mg once a day) therapy was initiated and after two-weeks the patient was in morphological remission and FISH analysis showed minimal residual disease with 1,5 % blast count. Dasatinib treatment was continued as consolidation therapy with methotrexate for four weeks, which resulted in CCyR. Dasatinib treatment was continued and HSCT was performed four months after diagnosis (July 2007). After HSCT no TKI therapy was used. Patient relapsed 1 year after the transplantation showing first cytogenetic relapse and a month later hematological relapse (August 2008). Patient started dasatinib therapy (70mg twice a day) with one cycle of high-dose chemotherapy and received CCyR. Since then dasatinib was continued as monotherapy and no additional cytotoxic drugs or donor lymphocyte infusions were given.

5.1.2 Cytogenetic characterization of the t(1;9)(q24;q34) translocation

G-banding and multicolor FISH analysis of the patient (Table 3) revealed a reciprocal translocation between chromosomes 1 and 9 (46,XY,t(1;9)(q24;q34) [5]/46, XY [3]), during diagnosis in 60% of the BM cells (Fig.10). Other translocations such as t(9;22) were undetectable. More specific analysis with probes specific for the 1p36 and 1q25 regions of chromosome 9 confirmed the translocation. The *BCR-ABL1* specific probe showed two green signals representing a normal *BCR* gene and three ABL1 signals. This indicated translocation between *ABL1* from chromosome 9 and an unknown gene situated in chromosome 1 in a region centromeric to 1q25.

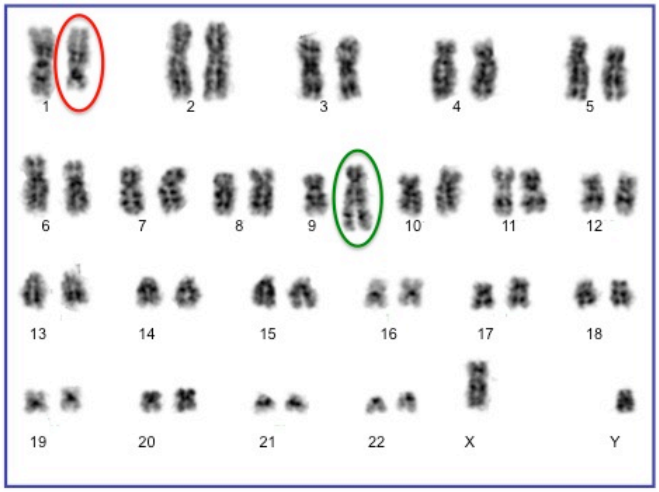

Figure 10. *Cytogenetic characterization of BM of a pre-B acute lymphoblastic leukemia patient (Patient #1, Table 3) with G-banding. Chromosome 1 is circled in red and chromosome 9 in green.*

5.1.3 PCR reaction and DNA sequencing of the *RCSD1-ABL1* fusion gene

As the *ABL* gene was involved in the 1;9 translocation, and the patient showed a response to dasatinib, ABL-driven leukemogenesis was suspected. According to a publication by De Braekeleer [263], who describes a t(1;9)(q24;q34) translocation gene in an ALL patient, with a possible involvement of *RCSD1* gene, a similar case was suspected in the present study. The molecular mechanism was examined in more detail and the possibility of *RCSD1-ABL1* fusion was explored by designing a specific forward PCR primer for *RCSD1* and a reverse primer for *ABL1*. The PCR reaction from patient #1, using diagnostic-phase cDNA, produced bands with sizes of approximately 500 bp (Fig. 11). Most of the bands included another neighboring band with similar size. PCR with negative and water controls did not yield any PCR products. The larger PCR product consisted of three first exons of *RCSD1* gene (Fig. 12) and in the smaller product the two first exons were included. In both of the cases *RCSD1* was fused to the exon 4 of the *ABL1* gene.

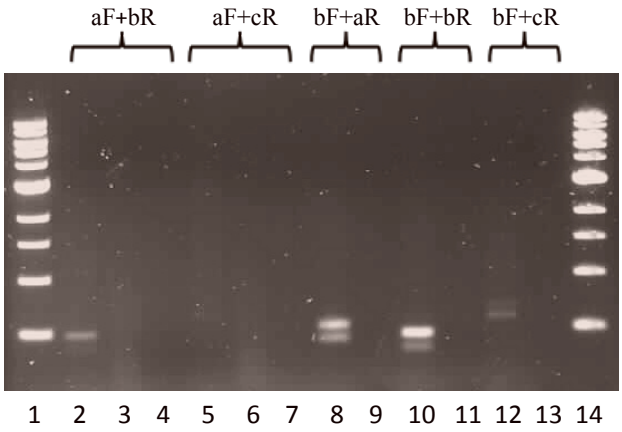

Figure 11. *Agarose gel of PCR reactions generated with forward RCSD1 and reverse ABL1 primers. Lanes 1 and 14: NEB 1 Kb DNA ladder; lanes 2, 5, 8, 10, and 12: PCR products with patient #1 cDNA; lanes 3 and 6: negative control cDNA; lanes 4, 7,9, 11, and 13: water control. aF, bF, aR, bR, and cR refer to primers listed in Table 4.*

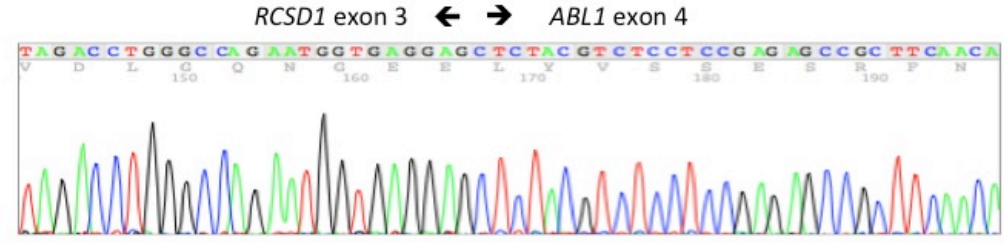

Figure 12. *The RCSD1-ABL1 fusion gene formed between RCSD1 exon 3 and ABL1 exon 4 (sequenced from a larger PCR product).*

5.2 Single cell phosphoprotein analysis (II)

The single cell phosphoprotein analysis method was chosen to study the immune cell function in CML patients at the diagnosis and during TKI therapy. This method is based on flow cytometry, which is able to measure phosphorylation levels of intracellular signaling proteins in distinct cell populations. *Ex vivo* cytokine stimulation was used to induce intracellular signaling activity.

5.2.1 Feasibility of single cell phosphoprotein analysis

The single cell phosphoprotein analysis method was optimized with healthy controls. The cell surface markers were able to discriminate monocytes, lymphocytes, and granulocytes from SSC vs. CD45 scatter and lymphocytes were further analyzed in the CD25 vs. CD4 scatter. The feasibility of the method was seen in the ability of the stimulating cytokines to induce cell specific intracellular signals. For example, GM-CSF was able to induce ERK1/2, STAT1, STAT3, and STAT5a phosphorylation in myeloid cells, but not in lymphocytes. A combination of cytokines IL-2, IL-10, and IFN-α was able induce phosphorylation of STAT5a, STAT3, and STAT1, while IL-4, IL-6, and INF-γ stimulated phosphorylation of STAT6, STAT3, and STAT1. The induced signals were most prominent in monocytes and less active in lymphocytes and granulocytes.

5.2.2 Basal phosphoprotein levels in CML patients

Although CML patients in the diagnostic phase have monocytes and granulocytes that are mostly Ph+ cells, the unstimulated basal phosphoprotein levels were similar to those of healthy controls (Fig. 13). This was also the case with STAT5a, although STAT5 signaling is known to be activated in Ph+ positive CML cell lines [264]. Basal phosphoprotein levels in Ph negative lymphocytes were also at the same level in both healthy controls and diagnostic CML patients (data not shown).

Patient samples studied during TKI therapy contained Ph negative cells since most of the patients had achieved CCyR at the time of sampling. STAT3 phosphorylation was decreased significantly in monocytes and granulocytes of dasatinib-treated patients (Fig. 13), as well as in lymphocyte subpopulations (data not shown). None of the other phosphoproteins levels studied differed significantly from those of healthy controls (myeloid cells, Fig.13, lymphocytes data not shown).

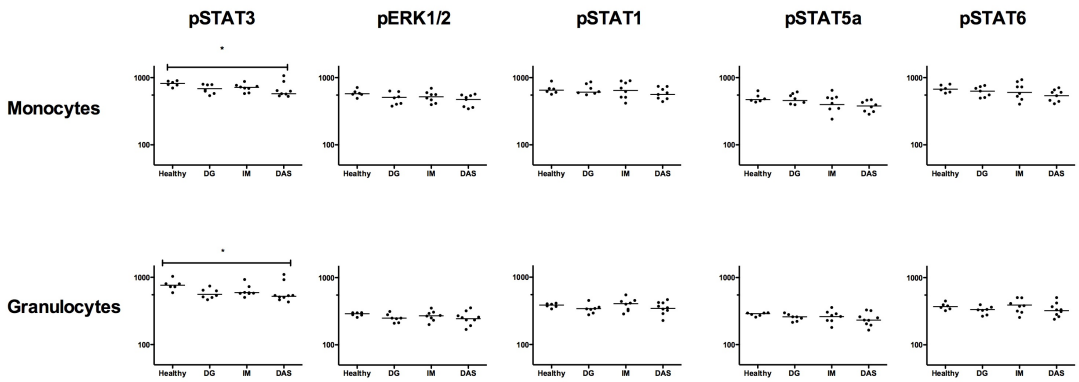

***Figure 13**. Baseline phosphoprotein levels in myeloid cells (monocytes and granulocytes) of healthy controls, and in CML patients at the time of diagnosis (DG), during imatinib (IM) therapy, and during dasatinib (DAS) therapy. *p<0.05*

5.2.3 Responsiveness of diagnostic and TKI-treated CML patients for *ex vivo* cytokine activation

GM-CSF induced strong signals through pERK1/2, pSTAT1, pSTAT3, and pSTAT5a in monocytes of healthy controls (Fig. 14). In monocytes of diagnostic-phase CML patients, the GM-CSF-induced signals were significantly lower in pERK1/2 (p<0.01), pSTAT1 (p<0.05) and STAT5a (p<0.05) (Fig. 14 A-C). The same trend was seen for the GM-CSF/pSTAT3 signal in monocytes (Fig. 14 D) and GM-CSF-induced signals in granulocytes, but the differences from healthy controls were not statistically significant (data not shown). However, the respective responses during imatinib and dasatinib therapy were corrected towards normal levels (Fig. 14)

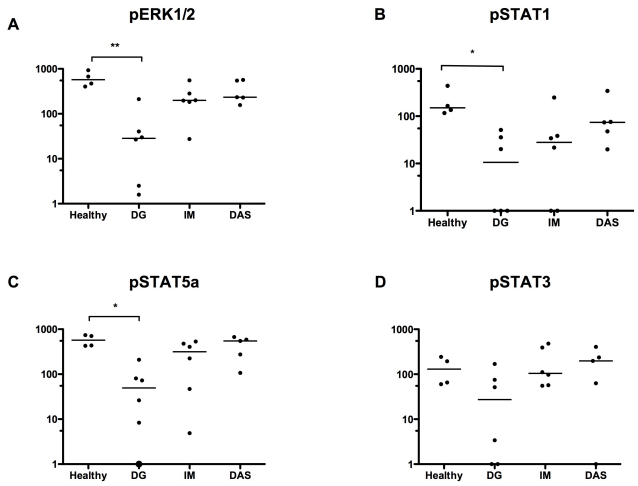

***Figure 14.** Intracellular pERK1/2, pSTAT1, pSTAT5a, and pSTAT3 responses to GM-CSF stimulation in monocytes of healthy controls, and in CML patients at diagnosis (DG) and during imatinib (IM) and dasatinib (DAS) therapy. *p<0.05, **p<0.01*

The same kind of unresponsiveness occurred in monocytes with other stimulations as well. The IFN-α/IFN-δ/pSTAT1 and IL-4/pSTAT6 responses were particularly lower in diagnostic-phase patients compared to healthy controls, but the difference was not statistically significant (data not shown). This kind of unresponsiveness was not seen during TKI therapy.

The lymphocyte fractions showed no statistically significant differences between healthy controls and diagnostic-phase CML patients, but interestingly, a couple of patients failed to respond to the IFN stimulus, as concluded from the STAT1 readout (Fig. 15 results from CD4+CD25+ cells). As in myeloid cell fractions, the lymphocyte populations responded normally to *ex vivo* cytokine stimulation during imatinib and dasatinib therapy (Fig. 15, results from CD4+CD25+ cells)

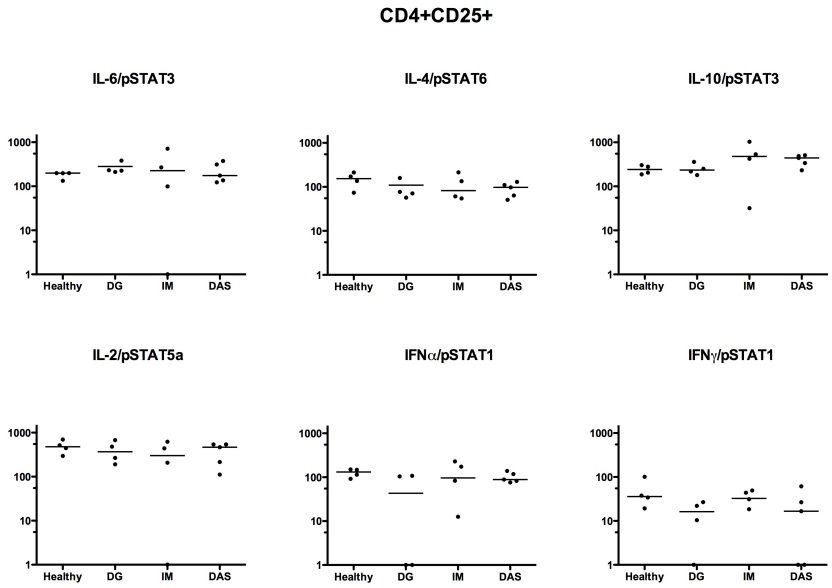

Figure 15. *Ex vivo cytokine responsiveness of CD4+CD25+ lymphocytes of healthy controls, and in CML patients at diagnosis, during imatinib (IM) and dasatinib (DAS) therapy.*

In order to reveal the cause of this unresponsiveness to GM-CSF stimulation in diagnostic-phase patients, the plasma GM-CSF concentrations were measured from representative samples as used in the *ex vivo* stimulations. However the GM-CSF levels did not differ between the groups studied (Fig. 16).

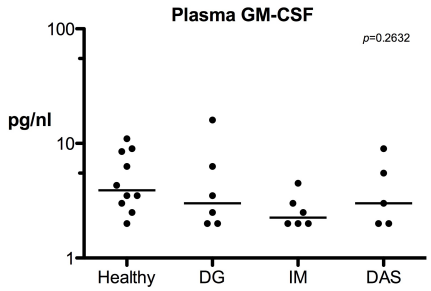

Figure 16. *Plasma GM-CSF concentrations in healthy controls, and in CML patients at the time of diagnosis (DG), during imatinib (IM) therapy and dasatinib (DAS) therapy.*

5.3 Automated FACS analysis software (III)

The FlowAnd analysis tool was developed to overcome the increasing workload associated with FACS analysis. As the FACS analysis platforms have increasing numbers of parameters and the amount of patient material can be huge, there is an increasing need for automated approaches to aid in the manual gating and complicated data analysis. In this project, the data already analyzed with manual methods in project II were utilized in the development of the FlowAnd system.

5.3.1 Feasibility of the FlowAnd system in FACS analysis

The manually gated data were compared with software assisted gating, which was based on three different clustering methods: SamSpectral, flowMeans, and mixture modeling. The cell debris resulting from dead leukocytes and particles of lysed red blood cells had to be filtered out before clustering the data. This was performed by setting a certain threshold in the SSCXCD45 scatter, because debris sediments near the origin and interferes with the clustering process. One patient data set (10 FCS files) was used in the comparison by gating according to CD45, CD4, CD3, and CD25 markers. An example of SSCxCD45 gating, comparing manual gating and mixture modeling, is presented in Figure 17.

The SamSpectral and flowMeans clustering methods resulted in clusters that were comparable, with biologically relevant populations, while mixture modeling resulted in too many clusters. The correlation of the manual gating and clusteration was highest with SamSpectral (0.99), while the flowMeans and mixture modeling had correlations values of 0.69 and 0.42, respectively. The time needed for clustering was lowest for flowMeans (1.5h) and SamSpectral (6h). The mixture modeling required 40h for the same analysis. Therefore, SamSpectral and flowMeans were superior to mixture modeling in many aspects.

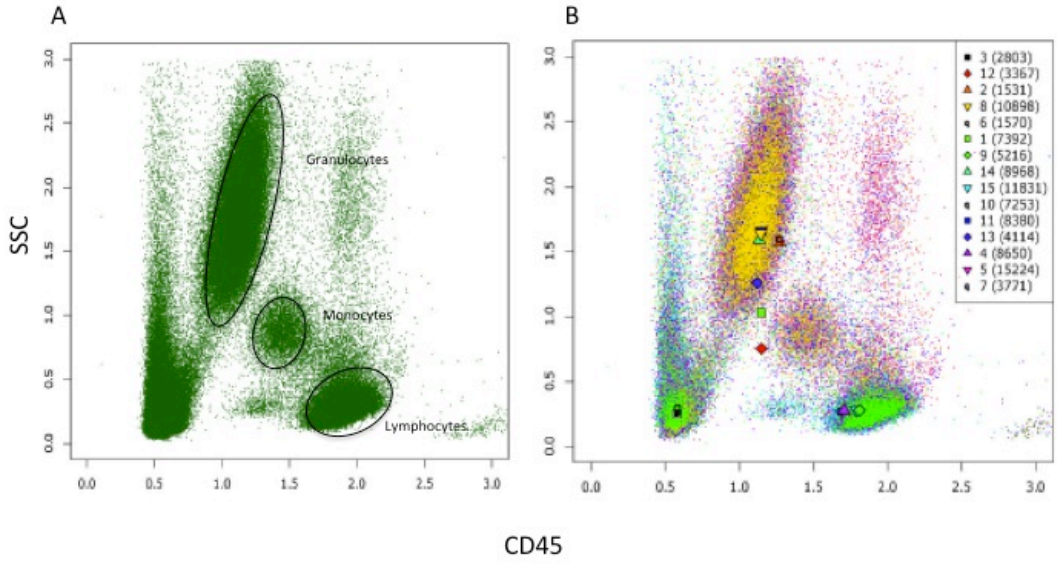

Figure 17. *Comparison of manual gating and software-assisted gating whole blood sample stained with CD45 antibody. A) Manual gating from SSCxCD45 plot with separated lymphocytes, monocytes, and granulocytes. B) Gating with FlowAnd software from the same sample, with a mixture modeling method.*

The performance of software-assisted gating with large-scale experiments was evaluated in a total of 74 FCS files from study II. In this setting, lymphocytes from SSCXCD45 scatter were gated and median baseline pSTAT3 values were determined. The clustering method used in this setting was flowMeans, which took 8.5 hours, with 20 min of manual work. The Kruskall-Wallis statistical test incorporated into FlowAnd analyzed the differences between healthy controls, diagnostic-phase CML patients, and patients receiving either imatinib or dasatinib. This resulted in a significantly lower level of STAT3 phosphorylation in the dasatinib group compared to the healthy controls, which was the same as the result obtained with manual analysis (data not shown).

5.4 Kinase target activation profiling (IV)

In order to characterize novel markers for the imatinib therapy response, kinase target activation profiles of BM MNCs derived from diagnostic-phase CML patients were analyzed with a phosphoproteomic array, which is able to measure the activation status of 46 different kinase targets from the same sample. CML is associated with deregulated cell signaling occurring by BCR-ABL1-dependent and independent mechanisms and many of the kinases involved in this process could be analyzed with this kit.

5.4.1 Activation of several kinases in diagnostic CML patients

The first analysis investigated the differences between healthy BM and BM affected with CML. All the samples were stored as snap-frozen pellets, as results from cells frozen with this method correlated well with fresh samples. Analysis of kinase target activation in healthy BM MNCs with the Proteome Profiler Human Phospho-Kinase Array Kit showed the highest phosphorylation levels for p53 S15, p70 S6 Kinase T421/S424, MSK1/2 S376/S360, GSK-3 α/β S21/S9, STAT1 Y701, TOR S2448, Akt T308, and RSK1/2/3 S380. However, the kinase activation profile of diagnostic CML patient BM MNCs differed from healthy controls, mostly due to phosphorylation of the p27 T157 (median ratio, CML patients/healthy controls, 3.148, $p<0.05$), C-Jun S63 (median ratio 2.194 $p<0.01$), Pyk2 Y402 (median ratio 2.006, $p<0.05$), p53 S46 (median ratio 1.421, $p<0.05$), Akt T308 (median ratio 1.386, $p<0.05$), and AMPKα2 T172 (median ratio 1.339, $p<0.05$) phosphoproteins (Fig. 18). The patient samples also showed higher variation between individual patients compared to the healthy controls (data not shown).

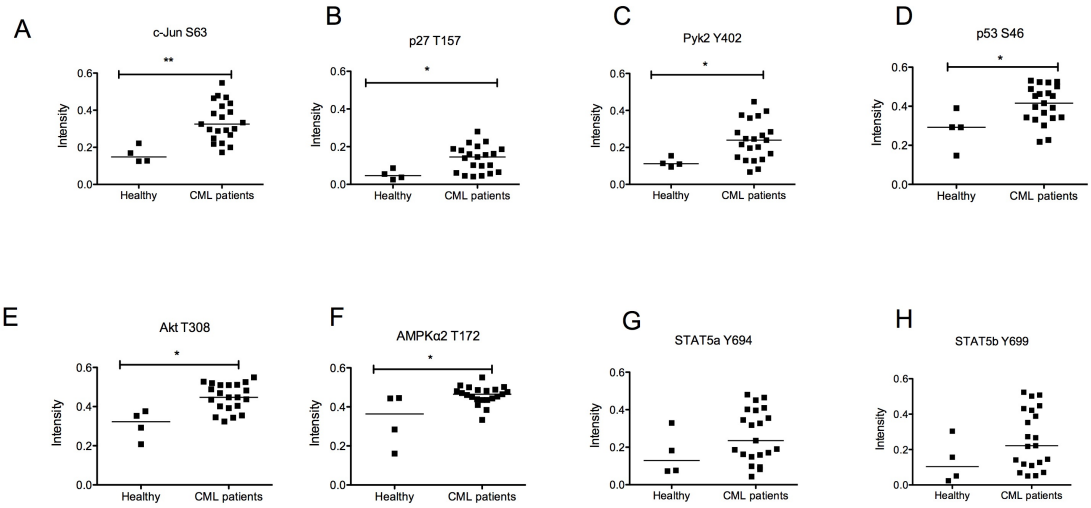

Figure 18. *Differences in the phosphorylation of signaling proteins in BM MNCs between healthy controls and diagnostic chronic myeloid leukemia patients. *p<0.05, **p<0.01.*

The K562 cell line was analyzed as a positive control, resulting in highly positive STAT5 phosphorylation, which was expected based on the current literature [264]. The phosphorylation was higher for the STAT5b isoform at site Y699 than for STAT5a Y694 (Fig. 19). However the STAT5 phosphorylation levels in CML patient BM MNCs were at normal levels, since comparison to healthy controls showed no statistically significant differences (Figs 18 G,H). Two other CML cell lines (EM-2 and MOLM-1) were analyzed and the STAT5 activation levels were weaker than with K562 (data not shown).

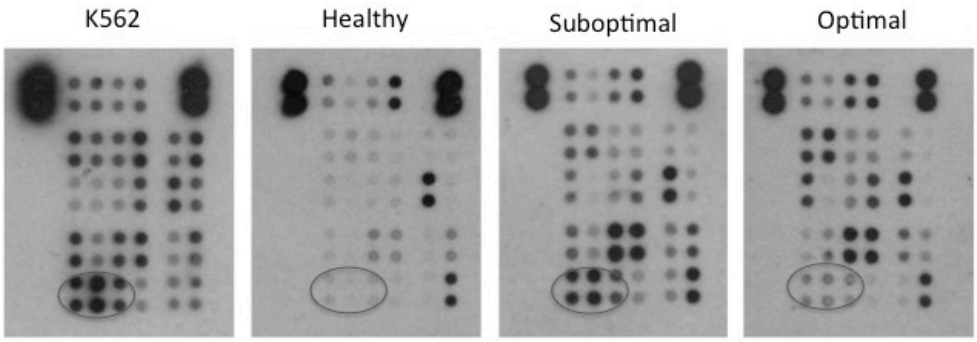

Figure 19. *Membrane A of the Proteome Profiler Human Phospho-Kinase Array Kit, representing K562 cells and BM MNCs analyzed from healthy control and diagnostic-phase CML patients with either suboptimal (Pt#15, Table 3) or optimal (Pt#6) response for imatinib. Circles indicate duplicate spots representing STAT5a/b (Y694/Y699), STAT5b Y699, and STAT5a Y694.*

5.4.2 Suboptimal response for imatinib is connected with activation of several kinase targets

A search for biomarkers for the suboptimal imatinib-response was performed by comparing BM kinase target activation profiles of diagnostic-phase patients divided into suboptimal and optimal response groups (Table 3). The patients in the optimal group were defined as achieving at least MMR after 18 months of imatinib therapy, while the suboptimal group achieved CCyR, but not MMR in the same time limit.

Analysis revealed 15 kinase targets with significantly elevated phosphorylation levels. The highest difference was visible in STAT5b Y699 phosphorylation (median ratio >3, $p<0.01$, Fig. 20A), while the differences in phosphorylation of the STAT5a Y694 isoform were not significant among the groups (Fig. 20H). The difference between these two STAT5 isoforms and optimal and suboptimal group are shown in Figure 19.

The following six proteins had a median ratio >1.5: PLC-γ1 Y783 ($p<0.05$), Pyk2 Y402 ($p<0.05$), Hck Y411 ($p<0.05$), Paxillin Y118 ($p<0.05$), p53 S15 ($p<0.05$) and RSK1/2/3 S380 ($p<0.05$) (Figs 20 B-G). Eight proteins had a median ratio below 1.5: RSK1/2 S221, p70 S6 Kinase T229, C-Jun S63, p53 S46, GSK-3 α/β S21/S9, Akt T308, p70 S6 Kinase T421/S424, and TOR S2448, while p70 S6 kinase T389 was significantly increased in the suboptimal group ($p<0.0342$), but the phosphorylation levels were at unreliable levels.

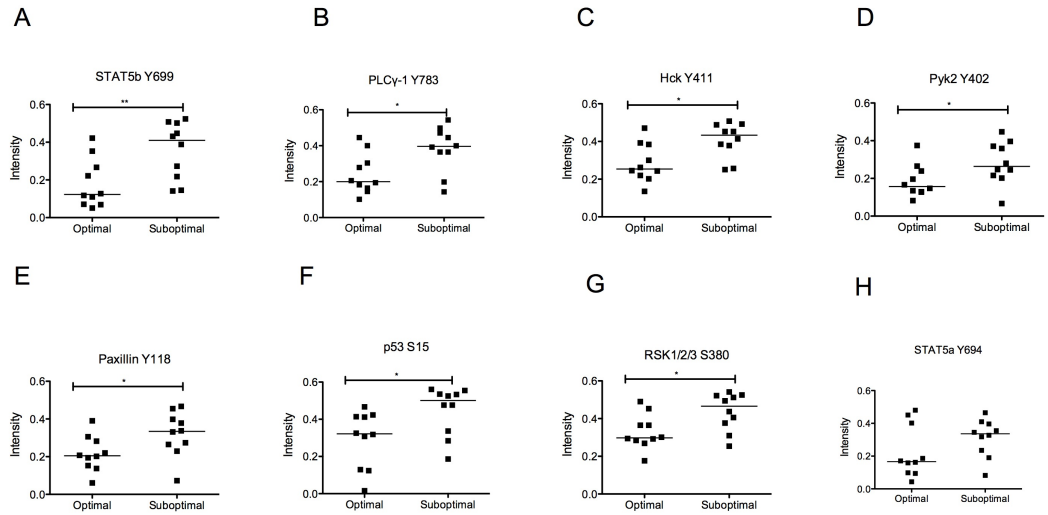

Figure 20. *Comparison of BM kinase target activities in diagnostic CML patients divided into optimal and suboptimal response groups. *p<0.05, **p<0.01*

Patients were also analyzed according intermediate and low risk Sokal factors (Table 3). The proteins that were statistically significant in optimal/suboptimal analysis were did not show statistically significant differences, but β-catenin protein expression and ERK1/2 T202/Y204 T185/Y187 phosphorylation were significantly (p<0.05) elevated in the intermediate group (data not shown).

5.4.3 Kinase target activation related to TKI resistance

Kinase activation related to TKI resistance was studied further by analyzing additional patients in whom TKI therapy had failed. Patient (#39) was analyzed as this patient carried an imatinib-resistant M351T mutation and showed no genetic response for imatinib, dasatinib and bosutinib. The patient had an exceptionally high HSP27 S78/S82 phosphorylation level (Figs 21A, B), which was not seen with any other CML patient or healthy control. The patient also had high STAT5b Y699 levels (Figure 21B).

Patient #40 had achieved a minimal cytogenetic response to imatinib and nilotinib and patient #41 was in blast crisis during BM sampling after a short period of imatinib-therapy. Results from these patients did not indicate high HSP27 phosphorylation, but their BM MNCs showed high expression of β-catenin and phosphorylation of ERK1/2, T202/Y204 T185 Y187, p70 S6 kinase T389, and p27 T198 (data not shown).

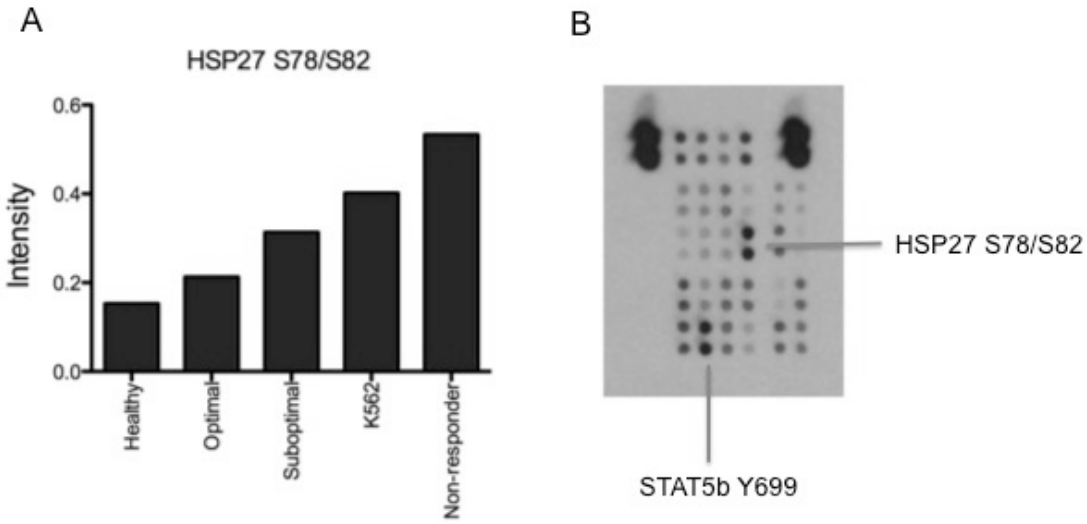

Figure 21. *Levels HSP27 S78/S82 phosphorylation of BM from patient #39 (Table 3). A) Comparison of HSP27 S78/S82 between different patient groups, patient #39, and the K562 cell line. B) Kinase array membrane with STAT5b Y699 and HSP27 S78/S82 highlighted.*

6 DISCUSSION

Imatinib was the first kinase inhibitor approved for clinical use and it dramatically changed the natural history of CML by inducing cytogenetic and molecular responses in a majority of patients. In the approximately 10 years since the introduction of imatinib, 12 small-molecule kinase inhibitors have been approved by the FDA to treat various types of cancer. However, experience in the clinic quickly showed emerging problems with resistance, mainly due to point mutations in the KD area. To overcome this drawback, second and third generation TKIs have been developed to treat imatinib-resistant and tolerant patients. Dasatinib and nilotinib have shown superior therapy responses in CML patients compared to imatinib, which has led to their approval as first line therapy modes in the USA and many European countries. Despite the excellent therapy responses seen with these two second generation drugs, persistent mutations such as T315I exist that are resistant even to dasatinib and nilotinib. Ponatinib is a third generation TKI specifically designed to target mutations resistant to second generation TKIs, including the notorious T315I mutation. Preliminary results from clinical trials with ponatinib have been promising. [179]

The clinical strategy of personalized medicine, which divides patients into subcategories according to certain clinical features and biomarkers in order to find optimal therapy modes, is evolving strongly into the field of TKIs. Experience in the treatment of BCR-ABL1-driven leukemias with TKIs, which spans over a decade, has illustrated that patients are individuals in terms of responsiveness. Extensive research has characterized many biomarker candidates for the imatinib response, based on genetic and proteomic approaches. However, although the repertoire of new TKIs is increasing, better characterization of patients is needed for optimal therapy selection. The aim of this PhD thesis was to characterize kinase activation statuses in CML patients, which could be connected with certain therapy responses.

The first study describes a pre-B ALL patient who was resistant to conventional chemotherapy normally used to induce a remission of leukemia. Cytogenetic characterization with G-banding showed a reciprocal translocation between chromosomes 1 and 9. More detailed analysis revealed that the *ABL1* gene had fused to an unknown gene. The patient received dasatinib, which had previously shown efficacy with Ph+ ALL patients. The dasatinib therapy, combined with methotrexate, rapidly resulted in CCyR and HSCT was carried out successfully. However, 12 months after the transplantation, the patient relapsed, but re-entered into CCyR following dasatinib therapy, confirming the reoccurrence of the same ABL –driven leukemia. Dasatinib caused some adverse effects, in the form of colitis and dermatitis and therapy was switched to imatinib, and patient has remained in remission since.

The good therapy response seen with a TKI aroused interest in the molecular mechanism underlying this disease. A similar case of an 11-year-old boy with pre-B ALL was recently described in a publication by De Braekeleer et al. The patient also carried a 1;9 translocation, which was shown to involve the *ABL1* gene. The group mapped the breaking point area of chromosome 1 by BAC cloning and FISH methods, and identified *RCSD1* as a possible fusion candidate [263]. Based on this information, specific PCR primers were designed in our project to bind the *RCSD1* and *ABL1* genes, which led to confirmation of the *RCSD1-ABL1* fusion gene. The *RCSD1* gene encodes for a CapZ-interacting protein (CapZIP), which is normally

expressed in immune cells, splenocytes, and muscle and is involved in actin filament remodeling. [265] The mechanisms by which *RCSD1* fusion to *ABL* induces leukemia is not known and it would be interesting to study whether RCSD1-ABL induces same kind of oncogenic signaling as is seen with BCR-ABL1.

This was the first time that an *RCSD1-ABL1* fusion gene was identified and confirmed by PCR. Most likely, the original publication suspecting *RCSD1* also involved the same fusion gene. Also three additional cases involving *RCSD1-ABL1* fusion gene have been reported in ALL patients.[104,109,266] One of these patients was treated with TKIs combined with dexamethasone and was able to receive a transient response.[266]. This finding and our results indicate the possibility of treating *RCSD1-ABL1* positive leukemias with dasatinib or other TKIs and suitability *RCSD1-ABL1* as a biomarker for TKI therapy.

The second study in this dissertation assessed immune cell function in CML patients at diagnosis and during TKI therapy, using single cell phosphoprotein analysis. This method, also called single-cell network profiling (SCNP), was originally developed by Garry Nolan's group at Stanford University. This FACS based method is able to assess signaling pathways in heterogeneous tissues such as PB and BM. Traditionally, measurement of cell phosphorylation has been conducted with Western blotting analysis, which uses lysates and thereby mixes all cellular contents. Phosphorylation levels obtained in this way are an average of all cell types and information regarding different compartments in heterogeneous tissues is lost. The single cell phosphoprotein analysis method is able to combine the benefits of traditional FACS analysis and Western blotting in multiparameter FACS by using cell surface markers and antibodies against phosphorylated intracellular signaling proteins. In order to evoke more prominent phosphorylation patterns, signaling can be induced with cytokine stimulation. [267]

This technique has been utilized in few studies that have evaluated its performance in the measurement of therapy responses in leukemia. A study conducted with AML patients revealed a correlation between therapy response for induction-chemotherapy and certain signaling patterns. The same study demonstrated reproducibility of the data obtained with single cell phosphoprotein analysis and emphasized its usefulness as a method for the discovery of novel prognostic factors for AML therapy responses. [268] A similar approach, also conducted with AML patients treated with valproic acid, all-trans retinoic acid, and theophylline, was able to cluster patients according to signaling activity that correlated with therapy responses.[269]

This is the first time that this method has been used to study immunity of CML patients. Controversy has surrounded the effects of TKI therapy on the immune cell function. The off-target inhibitory activity of TKIs affects many kinases involved in immune responses. Imatinib and dasatinib can inhibit T cell function *in vitro* [270-272]. On other hand, in the clinical trials assessing safety of TKI therapy, no remarkable occurrence of opportunistic infections have been observed, which argues against immunosuppressive effect of TKIs.

Different cytokines essential for regulating the immune system were used to stimulate intracellular signaling in fresh blood samples obtained from healthy controls, untreated diagnostic –phase CML patients, and patients treated either with imatinib or

dasatinib. The measurement of phosphoproteins STAT1, STAT3, STAT5a, STAT6, and ERK1/2 in different cell populations was made possible with multiparameter FACS. One major finding was that myeloid cells of diagnostic-phase patients are unresponsive to GM-CSF stimulation, which was reversed during TKI therapy. This was not explained by plasma GM-CSF levels. Deficient GM-CSF receptor function in malignant myeloid cells could explain the weak responses [273] and would implicate autonomy of leukemic cells. In general, no significant differences were noted between the healthy controls and CML patients treated with TKIs with respect to their phosphoprotein responses. This finding further argues against an immunosuppressive role of TKIs in CML patients.

The basal phosphorylation levels did not differ between healthy controls, or diagnostic phase and imatinib-treated CML patients in any of the cell populations analyzed. Dasatinib treatment was associated with low phosphorylation levels of the studied signaling proteins, particularly STAT3, which was significantly decreased compared to the healthy controls in both lymphoid and myeloid cell compartments. Although STAT3 is not a direct target of dasatinib, but transient dasatinib mediated STAT3 inhibition was noted in a study conducted with solid tumor cell lines and was likely mediated via SRC family kinase inhibition.[274] STAT3 has many cellular functions, and one of them is promotion of oncogenesis by upregulating expression of genes essential for survival, proliferation, angiogenesis, and metastasis. Another cancer promoting effect is suppression of tumor immunity by STAT3. [275] Inhibition STAT3 phosphorylation in dasatinib-treated patients might thus reinforce the anti-tumor immunity.

The purpose of study III was to develop a tool for automated FACS data analysis that would facilitate the laborious manual work. Multiparameter flow cytometry is now routinely used in clinical laboratories for diagnostic and disease follow-up purposes. Despite the technical improvements made in the last few years to increase the throughput of FACS instrumentation, the data analysis platforms have not reached their full potential. Data analysis is the most challenging and time-consuming step in flow cytometry and it is also a major source of variance among clinical tests [276]. Gating has traditionally been based on the personal intuition of the analyzing person and has lacked standardization. Manual gating also has limitations with the two-dimensional analysis setting. Automated approaches can process the data multidimensionally, thereby gaining novel information and also diminishing variation caused by manual gating.

The FlowAnd FACS analysis tool performed the full range of FACS data analysis and the results were comparable to those achieved by manual work. However, the FACS immunostainings analyzed this way were rather simple, and further development is needed to handle the current demanding routine analysis platforms. Previous similar approaches, such as FLAME [262], FIND [277], and flowCore[259], have been published before. However, FLAME was unable to handle the amount of data that existed in our study II data files. FlowAnd has a more diverse set of analysis tools than does FIND and it turned out to be more user friendly than flowCore.

Study IV continued with analysis of cell signaling associated with CML, with the aim of finding novel prognostic factors that could predict a response to imatinib at the time of diagnosis. MMR is considered as the therapy target in newly diagnosed CML

patients. This should be achieved within 18 months, in order to have an optimal treatment response. [233] It would useful to be able to predict the therapy response earlier, as there are different options regarding the TKIs available. Traditional clinical factors like Sokal, Hasford, and EUTOS are not able to provide an optimal prediction of the response to imatinib, and since research has identified many potential biological biomarkers, more effort is needed in this field.

Comparison of kinase activities of BM MNCs from diagnostic phase patients, divided into optimal and suboptimal groups according to their responses to imatinib, revealed elevation of several phosphoproteins associated with poorer outcome. The most marked difference between these groups was seen in STAT5b phosphorylation. The CML cell lines were analyzed as well, and showed highly different kinase activation patterns compared to patients' material, which points to the importance of using primary cells as research material.

The high variety of STAT5 phosphorylation levels seen in patient material was surprising because STAT5 is regarded as one of the main proteins mediating the BCR-ABL1 signaling cascade. This activation has been clearly demonstrated in many studies utilizing cell lines. [82,264] One of the most common experimental models used in CML research is the K562 cell line, which showed extremely high STAT5b phosphorylation, in our studies. The K562 line is derived from a blast crisis patient, who also had additional chromosomal changes, [278] so that this cell line probably does not reflect the situation in chronic phase CML patients. Studies conducted with patient samples indicate that STAT5 activation is associated with advanced phases of the disease. [279,280] The conclusion from these results is that STAT5 activation, and especially that of the STAT5b isoform, is linked to disease progression and may predict a poorer outcome at the time of diagnosis of chronic phase patients. In addition, three patients in the present study, who had failed with different modes of TKI therapy, showed signaling patterns that were not seen in imatinib-responsive patients, which could implicate further BCR-ABL1-independent survival mechanisms.

7 CONCLUSIONS

This thesis characterizes a novel *RCSD1-ABL1* fusion gene in an ALL patient that results from a translocation between chromosomes 1 and 9. The patient responded well to dasatinib, which is normally used in the treatment of BCR-ABL1 positive leukemias. These findings provide evidence that the indicated leukemia is caused by aberrant ABL1 kinase activity resulting from fusion to RCSD1, although the precise molecular mechanism is unknown. Despite only five ALL patient cases have been reported with the specific genetic abnormality, the findings serve a broader implication as they provide a novel tool for identification of ALL patients who will be responsive to dasatinib. This also gives emphasis on the importance of dividing patients into subcategories according to their genetic background for optimal therapy selection.

In addition to the *RCSD1-ABL1* fusion gene, this thesis also argues against an immunosuppressive role of TKIs in CML patients, as the responsiveness to various *ex vivo* cytokine stimulations remained at normal levels, as revealed by single cell phosphoprotein analysis of imatinib- and dasatinib-treated patients. However, the goal of connecting distinct signaling patterns to therapy responses was not achieved with this method. Myeloid cells of diagnostic-phase patients were found unresponsive to GM-CSF stimulation despite the fact that plasma levels of this growth factor were at normal levels. The findings may indicate an autonomous role of Ph+ leukemic cells in untreated CML patients, since phosphoprotein responses to GM-CSF stimulation were normalized with TKI therapy. The basal STAT3 phosphorylation levels were diminished in patients treated with dasatinib in all cell compartments and could be connected with dasatinib-induced anti-tumor immunity. The laborious FACS data analysis performed with this single cell phosphoprotein method led to the development of an automated FACS analysis program. This is likely a prototype for future automated FACS analysis programs needed to facilitate the arduous manual work.

Prognostic factors connected to certain imatinib therapy responses were searched from BM signaling activities in diagnostic phase CML patients. The phosphoproteomic approach was able to identify multiple signaling proteins that were associated with poorer outcome. The main phosphoprotein detected in this way was STAT5b, which has been shown to contribute to disease progression in CML.

This doctoral thesis provides novel information about different biological prognostic factors related to TKI therapy responses in the treatment of CML and particular Ph negative ALL. The findings can possibly be utilized in the future in clinical applications such as optimal therapy selection, risk assessment and the measurement of TKI therapy responses.

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